THE ROLE OF DOPAMINE AND SODIUM TRANSPORT INHIBITOR IN NATRIURESIS

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ABSTRACT

Endogenous sodium transport inhibitor (ESTI) and renal dopamine (DA) are thought to be important in maintaining sodium balance and in the pathogenesis of hypertension. In order to study the roles of these factors, methods for the measurement of ESTI were developed, based on the inhibition of Na, K-ATPase and digoxin-like immunoreactivity. Methods for the extraction of ESTI were studied and improved. An HPLC method for the measurement of DA was established and studies were done to find an appropriate preservative for urine DA.

The roles and interrelationship of ESTI and DA were studied in 4 cross-sectional and 4 longitudinal studies in humans. To define the roles of ESTI further, an animal study was done where DA production was inhibited by carbidopa in the rat.

In cross-sectional studies of healthy subjects there was no correlation between sodium excretion and ESTI but there was a significant correlation between sodium and DA excretion. However, in normotensive subjects with a family history of hypertension and in hypertensive patients, the sodium and DA correlation did not hold.

In 164 NIDDM patients grouped according to urine albumin excretion, DA excretion was lower and atrial natriuretic peptide (ANP) concentration was higher with increasing albuminuria. Dissociation between renin and aldosterone levels was also observed. ESTI was significantly higher in the hypertensive group.

In acute volume expansion induced by headout immersion and saline infusion in healthy subjects, no significant changes in ESTI and DA were found. The dopamine/noradrenaline ratio correlated with sodium excretion. In oral salt loading for 5 days in healthy subjects on a controlled diet, there was no significant increase in DA excretion despite of 9-fold increase in sodium excretion. Circadian variation of DA excretion was affected by dietary salt intake. When sodium excretion reached a

new steady state after 3 days of sodium supplementation, plasma ANP peaked and followed by an increase in plasma Na, K-ATPase inhibitor.

During salt loading in the rat, both ESTI and DA increased, but the increase in DA lagged behind by 1 day. When rats were treated with carbidopa, there was a 70% decrease in DA. ESTI increased in carbidopa-treated rats even on low salt. On day 5, there was a surge of ESTI in control high salt rats but this surge in the treated rats was seen on day 4.

In summary, both ESTI and renal DA have been found to be associated with changes in sodium excretion under different conditions. Changes in these two natriuretic factors by experimental manipulation did not affect sodium excretion. Under acute expansion of ECF volume, ESTI did not play a significant role. DA excretion correlated with sodium excretion under physiological conditions.

LIST OF ABBREVIATIONS

%I	Percentage of inhibition
β	Standardized coefficient of regression
°C	Degree Centigrade
µl	Microliter
µM	Micromole per liter
A	Adrenaline
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
CATS	Catecholamines
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
Cr	Creatinine
CV	Coefficient of variation
d	day
DA	Dopamine
DBP	Diastolic blood pressure
DHBA	Dihydroxybenzylamine
DLI	Digoxin-like immunoreactivity
DNA	Deoxyribose nucleic acid
EA	Enzyme acceptor
ECD	Electrochemical detector
ECF	Extracellular fluid
ED	Enzyme donor

EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol Bis (β-aminoethylether) N,N,N',N' tetraacetic acid
ESTI	Endogenous sodium transport inhibitor
g	gram
G6PD	Glucose-6-phophate dehydrogenase
GFR	Glomerular filtration rate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
h	hour
HbA_1	Hemoglobin A_1
HCl	Hydrochloric acid
HEIA	Homogenous enzyme immunoassay
HPLC	High performance liquid chromatography
IC_{50}	Ouabain concentration required to cause 50% inhibition
IDDM	Insulin dependent diabetes mellitus
KCl	Potassium chloride
LD	Lactate dehydrogenase
M	Mole per liter
MAP	Mean arterial pressure
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Milliliter
mM	Millimole per liter
mmol	millimoles
mV	Millivolt
NA	Noradrenaline
Na, K-ATPase	Sodium, potassium activated adenosine triphosphatase
NaCl	Sodium chloride
NADH	Reduced nicotinamide adenine dinucleotide
NIDDM	Non-insulin dependent diabetes mellitus

nM	Nanomole per liter
nm	Nanometer
NO	Nitric oxide
OE	Ouabain equivalent
OLI	Ouabain-like immunoreactivity
PEP	Phosphoenolpyruvate
PGS	Prostaglandins
PK	Pyruvate kinase
PNPP	Para-nitrophenylphosphate
r	Coefficient of correlation
RCM	Radial compression module
RIA	Radioimmunoassay
RNA	Ribose nucleic acid
SBP	Systolic blood pressure
SD	Standard deviation
sec	Second
SEM	Standard error of the mean
SSBP	Supine systolic blood pressure
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
UAE	Urinary albumin excretion

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INTRODUCTION

Approximately one-third of the total body water is contained within the extracellular fluid (ECF) compartment. This space provides the cells with essential nutrients and contains the intravascular volume which continually renews the supply of cellular nutrients. Maintenance of an appropriate ECF volume is essential for the homeostasis of body functions. Sodium is the principal cation in the ECF. The regulation of ECF volume is through regulation of renal excretion of sodium. The mechanism of this regulation is complicated and accomplished by a combination of factors, including systemic and local hormones.

Essential hypertension, a primary increase in blood pressure (BP), is a common disorder affecting 10-15% of the adult population. It affects almost every organ system in the body, resulting in high morbidity and mortality. In a proportion of hypertensives, BP is ECF volume dependent. It is therefore important to understand the pathogenesis of this group of patients so that proper treatment and even preventive measures might be implemented.

Dietary salt intake is one of the many factors which has been suggested to predispose the development of high BP. Intervention studies have shown that decrease in sodium intake will cause a reduction of BP in hypertensive patients (Morgan *et al* 1987). Epidemiological studies add further support to the role of salt in hypertension. Review of data from 27 human populations shows a strong correlation between the average salt intake and BP (Glieberman 1973). The prevalence of hypertension increases when salt intake is more than 2 grams per day (Berglund 1983). An international cooperative study of electrolyte excretion and BP, the INTERSALT study, confirmed that sodium intake is related to BP in individuals and to the rise of BP with age (Rose *et al* 1989). Populations with a very low sodium intake are associated with minimal rise of BP with age and near absence of hypertension.

There is strong evidence to indicate that the kidney plays an important role in the pathogenesis of salt-induced hypertension. In animals, excessive dietary sodium can

raise BP especially when renal function is impaired (Haddy 1980). A proportion of normotensive animals (e.g. rats) become hypertensive on a high salt diet. Furthermore, these animals can be made more sensitive to salt if their renal mass is reduced. Dahl was able to raise inbred strains of rats that develop hypertension without reduction in renal function on a normal or high salt diet. When kidneys of these salt sensitive rats were transplanted into normal rats, the BP increased in the recipients (Dahl & Heine 1975). In man, reduction in renal mass is associated with a reduction in the capacity for natriuresis and is often associated with hypertension (Haddy 1980). A low salt diet is often effective in reducing hypertension in end stage renal failure, thus supporting a role for salt in the genesis of hypertension in renal failure. Diuretics promoting natriuresis can lower BP in many hypertensive patients, especially in those with low plasma renin activity. Finally, as in the salt sensitive rats, hypertension in humans can be corrected by transplantation of a kidney from a normotensive donor (Curtis *et al* 1983).

Dahl *et al* suggested that a circulating substance might be involved in the genesis of hypertension in salt sensitive rats (Dahl *et al* 1969). This hypothesis was later extended to include the rise of BP in experimental hypertension (Haddy & Overbeck 1976). Blaustein put forward a hypothesis to explain the mechanism by which a rise in circulating sodium transport inhibitor may cause a rise in BP by inhibiting sodium-calcium exchange (Blaustein 1977). De Wardener and MacGregor later proposed a model which also included a role for the kidney in the pathogenesis of induced hypertension (de Wardener & MacGregor 1980). According to this hypothesis hypertensive patients are predisposed with a renal defect, and when sodium intake exceeds the renal excretion capacity, the rise in intrathoracic blood volume accompanying the increased ECF volume and plasma volume triggers the release of an endogenous sodium transport inhibitor (ESTI). This inhibitor facilitates natriuresis by inhibiting tubular sodium reabsorption. The high circulating levels of ESTI not only inhibit renal sodium reabsorption, but also sodium transport in other tissues including the blood vessels. Such an inhibition will lead to increase in intracellular calcium in the blood vessels, as suggested by Blaustein, resulting in a rise in peripheral resistance, and thus hypertension. Although there is a large volume of literature

supporting the presence of ESTI, its identity has not been elucidated even after many years of intense research efforts (Woolfson *et al* 1994). Furthermore, there are conflicting reports that do not support the presence of ESTI during ECF volume expansion.

In this hypothesis of pathogenesis of hypertension, a genetic or acquired deficiency in natriuresis plays a critical role in the development of hypertension. However, the nature of the renal defect is unknown. Lee suggested that the fault might lie in a failure to mobilize dopamine (DA) in the kidney in the presence of increased dietary salt (Lee 1987). DA is formed in renal tubular cells from circulating L-dopa (Lee 1982). Formation of renal DA results in vasodilatation of renal blood vessels, by action on vascular receptors. Furthermore, natriuresis is enhanced by an effect on vascular sodium transport mechanisms. There is evidence to show that renal DA production increases in response to oral sodium load in both man and animals. A failure of DA production in response to sodium challenge in some hypertensives has been shown. Ethnic groups that have a high prevalence of hypertension fail to increase DA. However, later studies showed that the amount of DA production was not in parallel with the increase in ECF volume (Cuche *et al* 1983, Jeffery *et al* 1989).

In this thesis, literature on the different factors affecting natriuresis, de Wardener's hypothesis and the DA theory are reviewed. This is followed by a description of the development of a sensitive semi-automated method for measuring ESTI and a high pressure liquid chromatographic method with sensitive electrochemical detection to measure DA. The inter-relationships between the sodium excretion, ESTI and DA were investigated in cross-sectional studies of healthy individuals, hypertensive patients, and non-insulin dependent diabetic (NIDDM) patients. The NIDDM patients with proteinuria represented a population with reduced renal tubular function, and thus reduced capacity for renal DA production. The roles of ESTI and DA in healthy subjects undergoing ECF volume expansion were studied. Volume expansion was induced by different methods: water immersion, saline infusion and oral salt loading. Major emphasis was on the oral salt loading method since dietary sodium intake is the common route for expanding the ECF volume. Ethnic differences in DA

response to sodium challenge have been reported (Critchley *et al* 1989). The present studies were conducted in Chinese, about whom there have been no previous reports. Finally, the role of ESTI was also examined in rats when the DA production was inhibited by carbidopa.

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CHAPTER 1

REVIEW ON SODIUM EXCRETION

RENAL EXCRETION OF SODIUM AND ITS REGULATION

I. SODIUM EXCRETION

The renal excretion of sodium is the major regulatory mechanism in the maintenance of the extracellular fluid (ECF) volume. Following ultrafiltration of the plasma through the glomerulus, the epithelial cells that form the individual nephrons selectively absorb the important components back to the circulation, for example: glucose, sodium, amino acids etc. (Briggs *et al* 1990, Kirchner & Stein 1994). Unabsorbed metabolites pass through the lumen and are excreted in the form of urine. Approximately 180 liters of fluid is filtered daily and approximately 178 liters are reabsorbed. For sodium, about 22,500 mmol are filtered daily and about 22,300 mmol are reabsorbed. The sodium left in the lumen is excreted in the urine. There are many mechanisms for sensing the status of the ECF volume. These sensors are responsible for triggering integrated neural and hormonal factors to influence the rate of reabsorption of sodium in order to maintain a constant amount of body sodium and an effective ECF volume.

In healthy people, sodium excretion can vary from less than 1 mmol per day to more than 500 mmol per day, according to dietary intake (Briggs *et al* 1990). Among the 52 populations examined in the INTERSALT study, the median for daily renal sodium excretion ranged from 0.2 mmol to 242 mmol (Elliott *et al* 1989). However, with the administration of intravenous saline, an excretion of up to 200 mmol/h can be achieved depending on the amount of saline infused. The mechanisms controlling natriuresis are able to cope with this large variation in sodium intake.

It has been recognized that the sodium regulatory system operates with a remarkable degree of sluggishness and appears to require the existence of rather large error signals (Briggs *et al* 1990). When an acute load of water is given to a healthy individual, 80% to 90% of this water load can be excreted through the kidney in a few hours. However, when an equal load of isotonic saline is given, it takes many more

hours to eliminate this load. For example, it was reported that only 15% of infused sodium was excreted during the first 3 hours and the excretion of all the sodium load took 48 hours (Drummer *et al* 1992).

An increase in daily sodium intake is associated with a transient period of positive sodium balance and the total body sodium rises. With time, sodium balance is re-established at a new steady-state level of increased total body sodium. Sodium excretion is adjusted to balance the amount of sodium intake. The time required to achieve balance varies from 1 to 9 days, depending on the amount of change in sodium intake (Briggs 1990). When sodium intake was restricted from 133 mmol/day to 10 mmol/day, sodium excretion was reduced and it took 4 days to reach a new steady state (Sagnella *et al* 1990). However, when sodium intake was increased to 350 mmol/day, the new steady state was achieved in only 3 days.

Sodium excretion is affected by numerous mechanisms with a substantial degree of redundancy and overlap (Briggs *et al* 1990, Rabkins & Dahl 1993). In general, failure of a single factor does not cause marked long-term deviations of salt balance. This has presented a fundamental problem in studying the effect of individual mechanisms. It is very difficult to dissect out only one factor and study its effect on sodium excretion.

II. CELLULAR MECHANISM OF SODIUM REABSORPTION

Reabsorption of the filtered sodium is accomplished by the transport of sodium from the apical membrane to the basolateral membrane of the epithelial cells lining the lumen. The apical membrane faces the lumen and the basolateral membrane is in contact with the interstitial fluid. On the basolateral membrane, the sodium-potassium active pump (the sodium-potassium adenosine triphosphatase) is the major system transporting sodium out of the cells into the circulation (Jørgensen 1986). The sodium pump utilizes energy derived from the hydrolysis of adenosine triphosphate (ATP). Sodium is transported out and potassium is transported into the cell with a stochiometry of 3:2 and an electrochemical gradient for sodium ions across the cell membrane is created. This gradient is the driving force for the sodium entry to the interior of the cell through specific channels located on the apical side of the cell membrane. On the apical membrane, there are transport proteins in the forms of channels, carriers, exchangers, or cotransporters. These transport proteins utilize the potential energy of existing electrical or chemical gradients to move sodium from the lumen into the cells. Examples of these transport mechanisms are: sodium-glucose cotransport and sodium-potassium-chloride cotransport on the loop of Henle; sodium channels located on the distal nephron and the collecting duct; and sodium-hydrogen exchange on the proximal tubules.

Regulation of the cellular transport of sodium in the nephrons can be brought about by a combination of several methods: kinetic factors, process of endocytosis / exocytosis, action of second messengers and long-term adaptation (Stokes 1994).

Kinetic factors

Kinetic factors involve any process increasing or decreasing the transport rate by changing the sodium concentration available to the apical membrane. For example, increasing the glomerular filtration rate (GFR) will expose the proximal tubule with increasing amount of sodium. This can increase the entry of sodium into the cell on the apical membrane and will increase the rate of sodium extrusion by the sodium-

potassium pump on the basolateral membrane. This mechanism is one reason why proximal tubular absorption of sodium exhibits flow dependency.

Endocytosis and exocytosis

This type of regulation requires the integrated action of the cytoskeleton. When the rate of reabsorption is low, some transporters in the apical membrane are sequestered in vesicles just below the surface. Upon appropriate stimulation, the subapical vesicles are inserted into the apical membrane, where they fuse to expose the transport proteins to the lumen. The increase in the number of available transporters increases the rate at which sodium can be transported into the epithelial cells. The stimulus activates both exocytosis and endocytosis so that the cycling of vesicles increases. Withdrawal of the stimulus reduces the exocytosis so that the vesicles reside in a quiescent state beneath the apical membrane ready for the next stimulus.

Second messengers

The largest and most diverse category of transport regulation involves the action of second messengers. The binding of hormones to specific receptors on the surface of the membrane can elicit the activation of second messengers. Examples of second messengers are cytosolic calcium, cyclic AMP, cyclic GMP, and membrane phospholipid-derived messengers. These messengers can directly affect ion channel activity, activate protein kinases that modify transporters, and alter cell pH to render transporters more or less sensitive to regulation by other agents. An important feature of regulation by second messengers is that many phenomena involve the concordant regulation of both apical and basolateral transporters.

Long-term adaptation

Most steroid hormones, for example aldosterone, exert their effects by long-term adaptation. Through activation of the specific receptor proteins complexing the nucleus, specific proteins, messenger RNA etc can be synthesized in the target cells. The permeability of the apical membrane to the entry of sodium can be altered to affect the transport rate. The rate of protein synthesis can be changed to regulate the number of functioning sodium channels or cotransporters on the apical membrane and

the number of sodium pumps on the basolateral membrane. Finally, the metabolic capacity of the epithelial cells can also be affected. These processes together serve to produce a sustained effect for the transport of sodium.

III. SENSORS MONITORING ECF VOLUME

The ECF space consists of the interstitial compartment and the intravascular compartment. There are sensors in both compartments to monitor the ECF volume. These sensors detect "effective" ECF volume rather than the absolute volume (Kirchner & Stein 1994). Effective ECF volume can be defined as the ratio of the ECF volume to the holding capacity. Thermoneutral, head-out water immersion procedure illustrates this (Epstein 1992). This experimental procedure involves a subject sitting in a tank of water filled up to the neck and it results in a sustained and marked increase in natriuresis without any change in the absolute ECF volume. It is associated with increases in cardiac preload, thoracic blood volume, cardiac output, and left atrial pressure.

Different receptors have been identified as the sensors for monitoring ECF volume (Briggs *et al* 1990; Kirchner & Stein 1994). They are the :

1. intrathoracic low-pressure receptors,
2. arterial baroreceptors,
3. carotid sinus receptors,
4. intracranial (or cerebrospinal) volume receptors,
5. intrarenal baroreceptors,
6. intrahepatic volume receptors, and
7. tissue receptors.

Some of these receptors trigger specific responses to control sodium excretion. For example, the intrathoracic low-pressure receptors are associated with the sensing of atrial stretch and are involved with the release of atrial natriuretic factor. The intrarenal baroreceptors are also involved in the release of renin and aldosterone in regulation of natriuresis. However, the significance of these receptors in regulating ECF volume under normal physiological conditions is controversial. Studies have given conflicting results because variations in the experimental designs where subjects have been subjected to extremes of volume status. In some studies where the subjects were suffering from different disease conditions that might affect the interpretation of the results. Furthermore, these receptors are probably acting together to achieve the

important task of monitoring ECF volume. It is almost impossible to dissect out any one of them and study its effects alone.

IV. FACTORS AFFECTING NATRIURESIS

Maintenance of the ECF volume is under the control of many complicated systems consisting of many factors. These factors include flow and pressure, the nervous systems, and the endocrine and parenteral systems (Briggs *et al* 1990; Kirchner & Stein 1994). They can also be classified into renal and extrarenal factors.

Renal factors

Glomerular filtration rate (GFR)

Glomerular filtration rate has been listed as one of the factors regulating natriuresis. Acute saline infusion and oral salt loading have been reported to increase GFR, leading to increase in sodium excretion (Briggs *et al* 1990). However,the classical experiment by de Wardener and colleagues demonstrated that the natriuresis of acute ECF volume expansion is partly independent of the GFR (de Wardener *et al* 1961). Even when the GFR of dogs was reduced by inflation of a balloon catheter located in the aorta proximal to the renal arteries, marked increase in natriuresis was still observed during intravenous saline infusion. Recent evidence suggests that GFR does not appear to be necessary for the fine tuning of salt balance. Changes in GFR are buffered by the glomerular-tubular balance and feedback mechanisms which are immediate and relatively precise adaptations of sodium absorption throughout the nephron (Briggs *et al* 1990; Kirchner & Stein 1994). These mechanisms are activated by changes in the postglomerular hemodynamics and peritubular capillary Starling forces. However, the presence of these mechanisms does not exclude a role for alterations in GFR in the control of natriuresis. In chronic volume expansion, the regulatory efficiency of their buffering capacity is suppressed (Briggs *et al* 1990).

Renal physical forces

Other than the GFR, renal physical forces such as the renal perfusion pressure, renal interstitial hydrostatic pressure, peritubular capillary hydrostatic pressure and peritubular oncotic pressure have been shown to play a significant role in controlling sodium excretion. These physical forces also influence one another to maintain

sodium homeostasis, thus regulating the ECF volume. For example, renal perfusion pressure can influence renal interstitial hydrostatic pressure as well as the peritubular capillary hydrostatic pressure (Kirchner & Stein 1994).

By changing the plasma protein concentration, peritubular oncotic pressure can be altered. There is sufficient data to support a correlation between large changes in peritubular oncotic pressure and proximal tubular sodium absorption during experimental maneuvers. However, its role is less important in physiological regulation of salt balance (Briggs *et al* 1990).

A technique of increasing the renal interstitial hydrostatic pressure without affecting renal blood flow or GFR has been reported (Haas *et al* 1988). Chronic implantation of iso-oncotic saline-albumin solution in a polyethylene matrix in the renal interstitium increases the renal interstitial hydrostatic pressure and induces marked natriuresis. On the other hand, reduction in the renal interstitial hydrostatic pressure by renal decapsulation significantly attenuated the natriuretic response to saline infusion (Garcia-Estan *et al* 1989). Thus changes in this physical force appear to be important in natriuresis.

Alterations in renal perfusion pressure markedly alter sodium excretion through mechanisms that are independent of changes in renal blood flow or GFR (Kirchner & Stein 1994). Its action is through the inhibition of sodium absorption. Despite substantial efforts, neither the site nor the mechanism of pressure-induced changes in reabsorption has been identified (Briggs *et al* 1990). Increase in systemic blood pressure can increase the renal perfusion pressure and facilitate sodium excretion. In normotensive offspring of essential hypertensive parents, higher blood pressure was speculated to have compensatory effects to restore normal natriuresis when compared with offspring of normotensive parents (Turner & Reilly 1993). Increased renal perfusion pressure was reported to be responsible for the 'escape phenomenon' of mineralocorticoid administration (Hall *et al* 1984). When an electronic device was used to maintain a constant baseline renal perfusion pressure in experimental dogs undergoing aldosterone administration, there was positive sodium balance and the

escape phenomenon was not observed. When the electronic device was turned off, the renal perfusion pressure increased and sodium balance was restored as a result of increase in sodium excretion. It would be difficult to demonstrate the effect of this physical force in normal physiological conditions since it interacts with many other systems.

Finally, sodium excretion can be affected without changes in the measurable renal forces. During vasodilation or vasoconstriction of the renal vasculature, the same renal perfusion pressure will reach the postglomerular peritubular capillary, where it can affect the sodium reabsorption process and thus influence sodium excretion (Kirchner & Stein 1994). Vasodilation will decrease sodium reabsorption; while vasoconstriction tends to increase sodium reabsorption.

Sympathetic nervous system

The kidney receives a rich supply of sympathetic nerves and they are found along all parts of the arterial tree concentrated in afferent arterioles close to the glomerulus (Briggs *et al* 1990). In addition, both proximal and distal tubular cells are directly innervated with adrenergic nerve terminals. Micropuncture techniques have showed that the proximal convoluted tubule is the primary site affected by changes in renal sympathetic nerve activity. Expansion of the ECF volume, induced by either oral salt loading or intravenous saline infusion, is associated with a decrease in the renal sympathetic nervous activity and an increase in sodium excretion.

Biological actions of the sympathetic nervous system are mediated through the release of endogenous catecholamines, the neurotransmitters at the adrenergic nerve terminals. The major catecholamines acting on the kidney are noradrenaline, adrenaline and dopamine. There are 4 types of adrenoreceptors to mediate the biological effects of catecholamines: α_1, α_2, $ß_1$ and $ß_2$ (Jeffries & Pettinger 1989, Rabkins & Dahl 1993). Noradrenaline induces vasoconstriction through α_1-receptors which are postsynaptic. Presynaptic α_2-receptors inhibit noradrenaline release from sympathetic nerve terminals; while postsynaptic α_2-receptors can mediate

vasoconstriction. α_1-receptors are coupled to phospholipase C and their activation increase free intracellular calcium as the second messenger for biological actions. On the other hand, α_2-receptors are coupled to adenylate cyclase by inhibitory G proteins and activation of this class of receptors reduces cAMP which is the second messenger. Both $ß_1$ and $ß_2$ receptors mediate the inotropic and chronotropic effects of catecholamines. $ß_2$-receptors also mediate smooth muscle relaxation. Stimulation of presynaptic $ß_2$-receptors enhances noradrenaline release from the sympathetic nerve temrinals. Both $ß_1$ and $ß_2$ receptors are coupled via stimulatory G proteins to adenylate cylase. Thus activation of ß receptors elevates cellular cAMP levels. The major adrenergic receptor mediating vasoconstriction within the kidney is the α_1-receptor. Stimulation of this class of receptor on the proximal tubular cells increases water and sodium reabsorption leading to a decrease in sodium excretion. Activation of α_2-receptors reduces cellular cAMP concentration to counteract biological actions initiated by other hormones on the nephron. Stimulation of $ß_1$-receptors on the juxtaglomerular apparatus enhances renin release.

The interactions between the renal nervous system and the different ECF volume sensors are very complex. During an intravenous sodium load with isotonic and iso-oncotic dextran in saline, the decrease in renal nerve activity was completely abolished by denervation of sinoaortic baroreceptor and vagotomy (Morita & Vatner 1985). However, when hypertonic sodium solution was infused, the decrease in renal nerve activity was completely abolished with combined hepatic denervation and sinoaortic baroreceptor plus vagotomy (Morita *et al* 1991). Furthermore, an oral sodium load resulted in a decrease in renal nerve activity, which was completely abolished by hepatic denervation alone (Morita *et al* 1993).

Direct stimulation of the renal sympathetic nervous system demonstrates the different mechanisms affecting sodium excretion (Kopp & DiBona 1984). The lowest level of renal nerve activity only affects the renin response to other simultaneously occurring nonneuronal stimuli. Increasing the frequency of stimulation selectively increases renin secretion and then reduces urinary sodium excretion. At high frequencies of renal nerve stimulation, both GFR and renal blood flow are lowered to reduce sodium

excretion. This approach of studying the renal sympathetic nervous system can provide information under normal physiological conditions (Kirchner & Stein 1994).

Renal dopamine

Dopamine (DA)-containing nerves have been identified within the kidney. However, their functional importance has yet to be determined (Rabkins & Dahl 1993). On the other hand, DA is also produced by proximal tubular cells by the action of aromatic-L-amino-acid decarboxylase (EC 4.1.1.28 or commonly known as dopa decarboxylase) on dihydroxyphenylalanine (L-dopa) delivered to the kidney by the systemic circulation (Brown & Allison 1981, Wolfovitz *et al* 1993). Inhibition of the dopa decarboxylase by carbidopa leads to a significant decrease in urinary DA excretion (Ball & Lee 1977). Damage to the renal tubules in chronic renal diseases also results in a decrease in DA excretion (Casson *et al* 1983).

Biological actions of DA are mediated by specific receptors: DA_1 and DA_2 ((Felder *et al* 1989, Lee 1993). DA_1 receptors cause direct vasodilation through activation of adenylate cyclase. Activation of DA_1 receptors causes an increase in natriuresis without alterations in renal blood flow or GFR. DA_2 receptors cause indirect vasodilation by inhibition of noradrenaline release and also inhibit aldosterone production. When DA_2 receptors are activated, adenylate cyclase is inhibited. Both DA_1 and DA_2 receptors are found on the apical and basolateral membranes of the proximal tubule. Activation of the 2 receptors can inhibit the sodium pump activity on the proximal tubule. Inhibition of the sodium pump involves GTP dependent regulatory protein and the intracellular messenger cAMP (Bertorello and Aperia 1990). Thus, intrarenal DA can influence sodium excretion through both hemodynamic and tubular mechanisms (Kirchner & Stein 1994).

Dietary sodium appears to be a major regulatory factor in the control of renal DA synthesis (Alexander *et al* 1974, Ball *et al* 1978, Carey *et al* 1981, Lee 1993). There is a close relationship between sodium intake and DA excretion. Increased sodium intake increases DA excretion; while sodium depletion leads to a decrease in

DA excretion. Even under constant sodium intake, the use of DA receptor antagonists or dopa decarboxylase inhibitor reduces sodium excretion (Bass & Murphy 1990). It has also been suggested that the chloride ion is important in increasing DA excretion (Ball *et al* 1978). A recent study suggested that dietary salt loading increases L-dopa uptake across the basolateral membrane of renal tubular cells, thereby increasing the synthesis of DA in the cells (Wolfovitz *et al* 1993). It was also suggested that the proximal tubular reabsorptive capacity regulates the synthesis of DA (Eadington *et al* 1991). Other proposals on the regulation of DA synthesis include the tubular transport of sodium dependent aromatic amino acids, intratubular effect of sodium, or modulation by other factors affecting natriuresis such as atrial natriuretic factors (Lee 1993).

ECF volume expansion by intravenous saline infusion induces natriuresis and an increase in DA production in both man and animals (Bass & Murphy 1990, Lee 1993, Kirchner & Stein 1994). Furthermore, natriuresis was attenuated by both selective or nonselective DA receptor antagonists, or dopa decarboxylase inhibitors such as carbidopa or benserazide. Increase in dietary proteins leads to an increase in DA production and in turn affects sodium excretion (William *et al* 1986, Young 1990). Provision of the the essential amino acid tyrosine is crucial for the synthesis of L-dopa which determines the tubular formation of DA. Protein depleted rats displayed increased sympathetic nervous activity and diminished DA excretion. Supplementation of a low protein diet with tyrosine restored DA excretion to normal but without affecting the sympathetic nervous activity. On the other hand, protein enriched rats showed augmentation of DA excretion. Recently, increased dietary phosphate intake in rats was also reported to increase DA excretion when the salt intake was kept constant (Berndt *et al* 1993). It is of interest to note that the increase in DA excretion was not accompanied by increase in sodium excretion. Increased DA excretion was due to an increase in tubular synthesis of DA. The detailed mechanism responsible has not been identified.

There have been reports that endogenous DA does not contribute to the regulation of natriuresis under all conditions of sodium loading (Bass & Murphy 1990, Kirchner &

Stein 1994). There have also been reports that sodium excretion in animals put on normal or high salt diets was not affected by the presence of a DA receptor antagonist or dopa decarboxylase inhibitor. When anesthetized dogs were infused with hypoosmotic saline, they displayed an increase in sodium excretion independent of DA excretion. Similarly, in the presence of DA receptor antagonists, ECF volume expansion in dogs with infusion of isoosmotic saline failed to reduce sodium excretion. Furthermore, intravenous saline infusion in man treated with carbidopa failed to demonstrate a decrease in sodium excretion (Jeffery *et al* 1989). DA excretion increased only in the female laboratory rats in response to sodium challenge (Young 1990). Male rats in the same laboratory on high sodium diet had a paradoxial fall in DA production. An alternative hypothesis was proposed to explain these conflicting results from different research groups on the role of DA in the regulation of sodium excretion (Eadington *et al* 1991). Under some conditions, proximal tubular reabsorptive capacity regulates the synthesis of DA instead of the generally accepted view that sodium chloride regulates DA synthesis. More studies are necessary to clarify the role of renal DA in the regulation of sodium excretion.

The question whether renal DA interacts with other factors in the homeostasis of effective ECF volume remains controversial (Lee 1993). Activation of the DA_1 receptors by agonists can increase plasma renin activity depending on the dose of the agonists. On other hand, infusion of angiotensin II in physiological doses resulted in the reduction of renal DA and a decrease in sodium excretion. There were also conflicting reports on the interaction between DA and vasopressin in blocking the hydroosmotic effect of vasopressin on the cortical collecting duct in animals. The interaction between DA and atrial natriuretic factor in regulating sodium excretion has not been elucidated. More studies are necessary to understand the complicated interaction between DA and other factors.

Renin-angiotensin system

The renin-angiotensin system has been recognized as an important physiological regulating system in controlling sodium excretion (Briggs *et al* 1990, Rabkin & Dahl

1993, Kirchner & Stein 1994). Renin is released from the granulated cells near the juxtaglomerular apparatus. It acts on the glycoprotein angiotensinogen to produce a decapeptide angiotensin I. Angiotensin I is biologically inactive, but it is rapidly converted to active angiotensin II by the angiotensin converting enzyme in vascular endothelium especially in the lung. This octapeptide is further cleaved to form active angiotensin III and other smaller inactive fragments.

The release of renin is regulated by factors affecting sodium excretion. The important modulators are blood pressure, tubular sodium concentration, the sympathetic nervous system and angiotensin II (Kurtz *et al* 1990, Hackenthal *et al* 1990). Renin release responds inversely to changes in renal perfusion pressure, and a fall in pressure is associated with an increase in renin secretion. Increase in the sodium load delivered to the macula densa reduces renin release. Thus, a high salt diet and volume expansion are associated with low plasma renin activity. On the other hand, a low salt diet and volume depletion are accompanied by low sodium levels in distal tubular fluid, resulting in increase of renin activity. Low frequency stimulation of the renal sympathetic nervous system enhances the rate of renin secretion directly without any change in GFR and renal blood flow. Angiotensin II inhibits renin secretion in a negative feedback loop. This is a direct effect, not dependent on changes in renal hemodynamics or tubular transport. Other inhibitory agents are: vasopressin, somatostatin, endothelin, endothelium-derived relaxing factor, and adenosine A_1 receptor agonists. On the other hand, prostaglandins, PTH, glucagon, vasoactive intestinal peptide, adenosine A_2-agonists, and histamine can stimulate renin release. Substances that influence renin secretion directly control the secretion via a limited number of intracellular second messengers, for example, cAMP, cGMP or phospholipase C. The second messengers modulate intracelullar calcium levels. An increase in intracellular calcium is associated with decreased renin release, and reduced intracellular calcium enhances renin secretion.

Although some ambiguity still exists about the biological role of the heptapeptide angiotensin III, the bulk of evidence suggests that angiotensin II is the major if not the sole biological active compound (Humphreys & Lin 1988). Receptors for this peptide

hormone have been localized to glomeruli, renal cortex, and the outer strip of inner medulla overlying the vasa recta. It has the capacity to influence sodium excretion through different mechanisms (Kirchner & Stein 1994). It can directly stimulate the synthesis and release of the salt-retaining steroid hormone, aldosterone. It has a profound effect on systemic vascular resistance and thereby modulate effective ECF volume. Angiotensin II can cause constriction of the efferent arteriolar of the glomerulus and contraction of glomerular mesangial cells, thus affecting GFR. Finally it affects the proximal tubular reabsorption of sodium directly. Infusion of physiological doses of angiotensin II directly into canine renal artery showed that angiotensin II can stimulate sodium transport in the proximal tubule. The physiological significance of angiotensin II in controlling sodium excretion is well defined. When an angiotensin-converting enzyme inhibitor was infused into the renal artery of a sodium-depleted dog, a significant increase in sodium excretion was observed (McCaa *et al* 1978). This inappropriate loss of sodium occurred despite a fall in both GFR and blood pressure.

Aldosterone

The mineralocorticoid aldosterone plays an important role in the control of sodium excretion under conditions of alterations in dietary sodium (Briggs *et al* 1990, Rabkin & Dahl 1993, Kirchner & Stein 1994). It is synthesised by the zona glomerulosa cells of the adrenal cortex. Its action is to reduce sodium excretion by promoting sodium reabsorption at the late tubular segments, such as the cortical and medullary collection ducts. Aldosterone secretion is directly stimulated by angiotensin II, plasma potassium concentration, adrenocorticotrophic hormone, and serotonin and its secretion is inhibited by atrial natriuretic peptides and dopamine. Increased dietary sodium intake leads to reduced aldosterone activity and sodium excretion is enhanced; while sodium restriction leads to increased aldosterone activity and sodium excretion decreases.

The cellular actions of aldosterone in promoting sodium reabsorption are mediated by the synthesis of a unique protein. The hormone first binds to cytoplasmic receptor

and this hormone-receptor complex is translocated into the cell nucleus, where it binds to specific nuclear receptor and initiates transcription of RNA to produce this protein. This protein enhances the sodium permeability on the apical side. Increase in the intracellular sodium concentration leads to a secondary increase in sodium pump activity on the basolateral membrane.

Renal Prostaglandins

Prostaglandins (PGS) are a group of cyclic fatty acids produced throughout the body and possess a wide range of biological actions (Rabkins & Dahl 1993). They are paracrine hormones produced at or near their site of action. Like other hormones, PGS act through hormone-specific receptors. However, the cellular mode of action varies according to cell type and is not fully understood.

The kidney is a major site of PGS production, metabolism and action. Intrarenally formed PGS have long been considered to affect renal excretion of sodium and water (Briggs *et at* 1990). However, the evidence that PGS play a regulatory role in sodium excretion is conflicting (Kirchner & Stein 1994). Infusion of PGS into the renal artery of animals results in vasodilation and increase in sodium excretion. It is uncertain whether the administration of PGS via the renal artery mimics either the physiological concentration or anatomical location of endogenously produced PGS. Therefore, the interpretation of these findings is difficult.

Different approaches have been used to overcome this problem (Kirchner & Stein 1994). The production of renal PGS is correlated with sodium excretion. Nonsteroidal anti-inflammatory drugs, potent inhibitors of PGS synthesis have been used to study the effect on sodium excretion. The effects of PGS on sodium-transporting epithelia are studied in vitro. Despite all these efforts, the role of PGS in controlling sodium excretion remains highly controversial. For example, urinary excretion of PGS is increased in both saline infusion and salt deprivation. Inhibition of PGS synthesis can increase, decrease or has no effect on sodium excretion. PGS may have a role in the maintenance of renal homeostasis by interaction with other hormones (Rabkins & Dahl 1993).

Renal Kallikrein-kinin system

The kallikreins are a series of glycoprotein enzymes (Rabkins & Dahl 1993, Kirchner & Stein 1994). There are two major forms of kallikreins: the plasma and the glandular (or tissue) forms. A complete kallikrein system in the glandular form is found in the kidney and has been proposed to increase sodium excretion in response to expansion of the ECF volume. Activation of this system leads to release of the protease kallikrein which acts on the substrate kininogen to release kallidin, the renal kinin. Kallidin is subsequently converted to bradykinin by aminopeptidases. Kinins are degraded into inactive peptides by kininases I and II. The latter is also the converting enzyme responsible for the activation of angiotensin I. Kininase II is located in the proximal tubule in high concentrations, thus preventing delivery of circulating kinins to the distal nephron, the primary site of endogenous kinin activity.

The kallikrein-kinin system appears to increase sodium excretion and to have vasodilatory action (Briggs 1990, Rabkins & Dahl 1993, Kirchner & Stein 1994). Acute infusion of exogenous kinins into the renal circulation produces significant increase in renal blood flow, sodium and water excretion without affecting GFR. Urinary excretion of kallikrein correlates with sodium excretion during saline infusion or after mineralocorticoid adminsitration. However, like the problems associated with the study of prostaglandins described previously, these results do not necessarily prove that the kinins have a regulatory role in sodium excretion. Furthermore, inhibition of the kalikrein-kinin system by protease inhibitor or the kinin-receptor antagonist do not alter sodium excretion in normal or expanded ECF volume states. Available evidence is against a role for kinins in sodium excretion. More studies are required to show the direct action of kinins on sodium excretion.

On the other hand, there is evidence that the kallikrein-kinin system has an indirect role in modulating sodium excretion through interaction with other systems (Rabkin & Dahl 1993). There are complicated interrelationships between kinins and the renin-angiotensin-aldosterone system, vasopressin and the prostaglandins. The mechanisms of interaction are not fully understood. For example, kininase II inactivates kinins but

converts inactive angiotensin I to the active angiotensin II. Aldosterone has been shown to increase kallikrein excretion.

Extra-renal factors

Natriuretic peptides

In man, 4 different types of natriuretic peptides have been identified that play a significant role in the maintenance of ECF volume through sodium excretion (Jamison *et al* 1992). The atrial natriuretic peptide (ANP), a 28 amino acid peptide, is the major circulating type synthesized and released from the cardiac atria. The brain natriuretic peptide (BNP), containing 32 amino acids, is synthesized in the brain and the heart. Its plasma concentration is about six times lower than that of ANP. However, the clearance of BNP is ten times slower than that of ANP. The C-type natriuretic peptide (CNP) has 2 variants: a 22 amino acid peptide and a 53 amino acid peptide. The larger peptide is mainly found in the brain, while the smaller peptide can be found in the kidney. But CNP cannot be found in the circulation. Lastly, urodilatin, a 32-amino acid peptide identical to the C-terminal sequence of proANP, has been found only in urine.

Biological activities and regulation of these natriuretic peptides are mediated through 3 types of receptors: receptors A, B and C (Jamison *et at* 1992). Binding of the natriuretic peptides to receptors A and B stimulates membrane-bound guanylate cyclase, which catalyzes the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP is the second messenger for the biological activities of the peptide. Receptor C functions as a clearance receptor to remove natriuretic peptides from the circulation. The peptide-receptor C complex undergoes rapid internalization. Receptor C is recycled back to the surface while the ligand is hydrolyzed in lysosomes.

The most important stimulus for the release of ANP is atrial stretch (Brenner *et at* 1990, Cogan 1990). This can result from conditions that increase the effective ECF volume, for example: salt loading, head-down tilt, head-out water immersion, primary aldosteronism, the syndrome of inappropriate antidiuretic hormone, renal failure, or

congestive heart failure. ANP regulates the ECF volume by promoting sodium excretion through both direct and indirect mechanisms (Briggs *et al* 1990, Rabkin & Dahl 1993, Kirchner & Stein 1994). ANP increases the GFR by increasing the glomerular capillary hydrostatic pressure, through afferent arteriolar vasodilation and efferent arteriolar constriction. It also reduces the rate of apical sodium entry through the luminal amiloride-sensitive sodium channels at the inner medullary collecting ducts. These two actions involve the binding of ANP to receptor A located on the glomerulus and the collecting ducts, and mediated through cGMP. Therefore, they can be regarded as direct actions of ANP to promote sodium excretion by reducing sodium reabsorption. On the other hand, there are other actions of ANP on parts of the renal structure that do not have ANP receptors and the biological responses are not associated with production of cGMP. For example, ANP inhibits angiotensin II-induced sodium reabsorption at the proximal tubule; it inhibits renin and aldosterone secretion; and increases the hydrostatic pressure in deep nephrons resulting in unfavorable pressure gradients for sodium reabsorption.

BNP binds to similar receptors to ANP and has similar biological actions to ANP. However, the affinity of BNP to the receptor is lower. In human heart failure, the ventricle becomes more important than the atrium in the synthesis and release of BNP (Mukoyama *et at* 1991). The significance of this natriuretic peptide in the maintenance of effective ECF volume remains to be elucidated.

CNP binds to receptor B and has similar cardiovascular actions to ANP but lacks the renal actions (Clavell *et at* 1993). Since CNP is abundant in the brain and cannot be found in the circulation, it is suggested that CNP is a neuropeptide. Furthermore, its receptor is unique and different from that of ANP and it is suggested that CNP could represent another distinct regulatory system (Jamieson *et at* 1992). Further research is required to understand its significance in the homeostasis of ECF volume.

Urodilatin is a unique intrarenal natriuretic peptide (Goetz 1991). It binds to ANP receptors with the same affinity as ANP, activates guanylate cyclase and induces natriuresis and diuresis. Unlike ANP, urodilatin does not inhibit renin or aldosterone

secretion. Urinary urodilatin correlates with diurnal changes in sodium excretion and also correlates better with sodium excretion than plasma ANP (Goetz *et at* 1990, Drummer *et at* 1991). Thus, it has been considered the most important natriuretic peptide regulating natriuresis. However, more work is necessary before this can be accepted (Humphreys 1991).

Natriuretic hormone - the endogenous sodium transport inhibitor (ESTI)

More than 30 years ago, de Wardener postulated the presence of an endogenous natriuretic hormone (de Wardener *et al* 1961). This hormone increases sodium excretion during ECF volume expansion by direct inhibition of sodium reabsorption in the nephrons. Infusion of plasma from volume expanded animals can maintain profound and long-lasting natriuresis. Since then many studies have shown evidence for the existence of this putative natriuretic hormone (de Wardener & Clarkson 1985). de Wardener also hypothesized that this natriuretic hormone is related to the pathogenesis of essential hypertension in subjects with a renal defect. However, the identity and the biosynthetic pathway of this hormone are still poorly understood. The scientific community has been confused by the use of a non-specific biological action, sodium pump inhibition, to study this hormone (Wechter & Benaksas 1990, Goto *et al* 1992, Schoner 1992, Woolfson *et al* 1994). Furthermore, the use of a large variety of measurement methods and purification strategies has produced more confusion.

Different names have been used for this putative natriuretic hormone: the third factor, sodium pump inhibitor, endogeneous digitalis-like immunoreactivity, endogenous digitalis-like factor, ouabain-like factor etc. The ESTI should be distinguished from non-specific inhibitors of the sodium pump by its direct binding to the cardiac glycoside receptor, the sodium pump (Goto *et al* 1992). Although it has an inhibitory action on the sodium pump, DA should not be considered as the natriuretic hormone as it works through the activation of DA_1 and DA_2 receptors via second messenger cAMP.

More than 20 substances have been proposed as the natriuretic hormone. They include lipids, steroids, ouabain, digoxin, bufodienolides, ascorbic acid, lignans, urodienolone, hemin, quaternary ammonium or immonium compounds, peptides and unknown small molecular weight substances. A detailed listing can be found in recent reviews (Wechter & Benaksas 1990, Goto *et al* 1992, Schoner 1992, Woolfson *et al* 1994). Among all these compounds, only ouabain has been purified to homogeneity for structural analysis (Ludens *et al* 1991, Mathews *et al* 1991). However, ouabain does not show any significant natriuretic activity *in vivo* except at toxic supra physiologic doses (de Wardener & Clarkson 1985). Without the identification of the chemical structure of this hormone, it is only possible to understand this hormone by its putative physiological mechanism.

The tissue of origin of this hormone is not known with certainty. Steroid hormones are usually synthesized in the adrenals while peptide hormones are products of higher centers in the brain and of other endocrine tissues. Based on experiments on focal lesions and stimulation of the hypothalamus, intracerebro-ventricular infusions, and the use of tissue extracts, it has been proposed that the hypothalamus is involved in the production of ESTI (Woolfson *et al* 1994).

It has been suggested that the natriuretic hormone is not necessarily restricted to one molecule (Wechter & Benaksas 1990). The research focus has also shifted to emphasis on the complete purification and characterization of compounds which will inhibit the sodium pump in different measurement methods rather than a natriuretic compound (Woolfson *et al* 1994). It is of importance to understand the ESTI thoroughly as it may be related to the pathogenesis of essential hypertension, a disease affecting 10-15% of the world's population.

Vasopressin

Arginine vasopressin (AVP) is a 9 amino acid peptide secreted from the posterior pituitary gland in response to increased osomolarity or hypovolemia (Rabkin & Dahl 1993). Its major biological action is to increase water permeability throughout the collecting duct, thereby allowing concentration of the urine. Furthermore, this

peptide has effects on sodium excretion by affecting GFR and tubular sodium reabsorption.

AVP can specifically bind to two different types of receptors, V_1 and V_2 (Humphreys & Lin 1988). V_1 receptors are found in glomerular mesangium, renal vasculature, and medullary interestitial cells. Binding to these receptors leads to an increase in intracellular calcium, which mediates vasoconstriction and prostaglandin synthesis. V_2 receptors are located along the renal tubule, especially the thick ascending limb of Henle's loop and the cortical and medullary collecting duct. Binding of AVP to these receptors is linked to the activation of adenylate cyclase and the generation of cAMP. This is responsible for hydroosmotic effects of the hormone. Increase in circulating AVP concentration enhances sodium excretion through binding to V_1 receptors. AVP induces contraction of the glomerulus to increase GFR, hence increase in sodium excretion. Furthermore, activation of these receptors stimulates prostaglandin synthesis, which then act locally to decrease tubular sodium reabsorption.

Endothelins

The endothelins are a family of three peptides all containing 21 amino acids (Simonson & Dunn 1993). They are vasoconstrictors involved in the control of cardiovascular and renal function and act as neuromodulators. Although expression of mRNA has been reported in other tissues, endothelin-1 is produced mainly by endothelial cells, for example renal endothelial cells, glomerulus, and also renal tubular cells (Rabkin & Dahl 1993). It is the only form detected in the human circulation. It is secreted on the basal side of the endothelial cells (Remuzzi & Benigni 1993). Endothelin-2 is only identified in monkey kidney tissue culture and endothelin-3 is found in brain homogenates. Studies on the physiological actions of endothelins in controlling sodium excretion have been conducted mainly using endothelin-1.

A high affinity specific endothelin receptor has been identified (Rabkin & Dahl 1993, Remuzzi & Benigni 1993). Biological actions after the binding to the receptor are mediated by the activation of inositol-specific phospholipase C and the subsequent

increase in cytosolic calcium concentration. Furthermore, the receptor complex can also activate the release of arachidonic acid, depolarize the membrane, enhance alkalinisation of the cell interior, and inhibit the active sodium pump. This receptor has been found in a large variety of tissues. In the kidney, endothelin-1 binds to glomeruli, vasa recta bundles, papillae and the proximal tubules.

Endothelin-1 is a potent renal vasoconstrictor, 30 times more potent than angiotensin II (Rabkin & Dahl 1993). Excretion of endothelin is enhanced by a number of factors, for example: increased hemodynamic shear stress, transforming growth factor, interleukin-1, angiotensin II, vasopressin, and epinephrine. Its action in affecting sodium excretion is through the effect on GFR and tubular reabsorption of sodium. Although endothelin can be detected in the circulation, it operates as a local paracrine hormone more than through the systemic circulation (Remuzzi & Benigni 1993). Micropuncture techniques have shown that *in vivo* administration of endothelin can induce an increase in systemic blood pressure associated with renal vasoconstriction (Vercellotti & Tolins 1993). Glomerular capillary plasma flow is reduced, with increase in both afferent and efferent arteriolar resistances. The net effect is to cause 30% - 50% decrease in single-nephron GFR. This will reduce sodium excretion. On the other hand, endothelin-1 at picomolar concentration can act as a natriuretic hormone by inhibiting the ouabain-sensitive sodium pump in inner medullary collecting duct cells by a prostaglandin-dependent mechanism (Zeidel *et al* 1989). The systemic vasoconstrictor effect of endothelin, as reflected in the pressor response after *in vivo* administration, is preceded by a transient vasodilation that is most likely mediated by release of vasodilators (Vercellotti & Tolins 1993). Thus, the potent vasoconstriction action of endothelin is often guarded by opposing vasodilatory action of other factors, such as endothelium-derived relaxing factor, ANP and plasma renin activity.

With its potent vasoconstriction effect, endothelin may play a significant role in a number of renal diseases, for example: glomerular inflammation, cyclosporin-induced kidney disease and post-ischemic renal failure (Rabkin & Dahl 1993). However, its significance in the maintainence of ECF volume through controlling sodium excretion

under normal physiological conditions remains to be elucidated (Kirchner & Stein 1994).

Endothelium-derived relaxing factor or Nitric oxide

Endothelium-derived relaxing factor (EDRF) is a short-lived diffusible substance released by endothelial cells that mediates the effect of a number of vasorelaxants (Rabkin & Dahl 1993). The chemical nature of EDRF is controversial. Some believe that nitric oxide (NO) is the EDRF while others would consider it to be part of the EDRF molecule. The biosynthetic pathway and the mechanism of biological action of NO have been described (King & Brenner 1991, Vercellotti & Tolins 1993). It is derived from the terminal guanidino nitrogen atom of L-arginine by the action of a soluble enzyme NO synthase. NO produced by the endothelial cells diffuses to the nearby target cells, such as smooth muscle cells and glomerular mesangial cells. It stimulates the guanylate cyclase to increase the cellular cGMP concentration. cGMP activates protein kinase to dephosphorylate the myosin chains causing vasorelaxation. Release of NO is stimulated by a number of factors, such as: acetylcholine, arachidonic acid, endothelin, shear stress, and hypoxia (Rabkin & Dahl 1993).

NO is a potent vasodilator. Its role in modulating sodium excretion is studied by the use of NO synthase inhibitors *in vivo*, such as L-N-monomethylarginine or L-N-nitroarginine-methylester (Vercellotti & Tolins 1993). Inhibition of the NO system results in decreased GFR and renal vasoconstriction. Thus, the stimulation of NO production induces sodium excretion and inhibition of NO activity decreases renal sodium handling. In animal studies, the inhibition of NO synthase significantly elevates systemic blood pressure (Baylis *et al* 1990). This indicates that NO may play a significant role in counterbalancing other vasoconstrictors in the control of systemic blood pressure, for example, endothelin.

Other hormones

A number of other hormones with other primary functions in the gastrointestinal tract, the endocrine tissues or brain have also been reported to influence sodium excretion (Humphreys & Lin 1988, Kirchner & Stein 1994). Vasoactive intestinal peptide,

substance P, secretin, neuro-peptide Y, cholecystokinin octapeptide and parathyroid hormone have all been reported to increase sodium excretion; while calcitonin gene related peptide, insulin, neurotensin, somatostatin and melanocyte stimulating hormone have been reported to reduce renal sodium excretion. Their influence on sodium excretion is often observed under supra physiologic doses. Sometimes, these hormones may modulate natriuretic activity of other established factors, for example parathyroid hormone has recently been reported to modulate the action of ANP during acute volume expansion (Geiger *et at* 1992). The physiological role of these hormones in regard to sodium excretion remains to be elucidated.

V. CONCLUSION

The maintenance of a constant effective ECF volume requires very complicated mechanisms. The kidney regulates urinary sodium excretion so that the total body sodium remains constant. Under normal physiological conditions, sodium excretion is adjusted to the amount of dietary sodium intake. Ingestion of additional sodium will provoke retention of water by the release of vasopressin to preserve isotonicity. This will lead to an increase in ECF volume. These changes are constantly monitored by different sensory mechanisms, which in turn trigger a multitude of physical or hormonal factors to increase sodium excretion and restore effective ECF volume.

Different factors can affect sodium excretion through a large variety of pathways. We can therefore infer that the body has set a high priority to ensure the homeostasis of the ECF volume. Such a system design presents fundamental difficulties in understanding the functioning of indiviudual factors. It is extremely challenging, if not impossible, to dissect out an individual factor and study its contribution to sodium excretion. The use of inhibitors or antagonists for a factor may stimulate the action of another factor to maintain the efficiency of sodium excretion especially under normal physiologic situations. On the other hand, when acute or relatively large changes in ECF volume are used to study different factors, it is difficult to extrapolate the results to physiological situations.

This review shows that many areas concerning sodium excretion remain to be clarified. It is envisaged that with more research, more natriuretic factors may be discovered.

CHAPTER 2

MEASUREMENT OF ENDOGENOUS SODIUM TRANSPORT INHIBITORS

I. LITERATURE REVIEW

A large number of methods for the measurement of endogenous sodium transport inhibitors (ESTI) can be found in the literature. These methods were developed to assess the changes in ESTI in different physiological / pathophysiological conditions and to monitor the progress of the purification procedures. However, all these methods suffer from a common problem that the identity of ESTI is still unknown. No standard preparation can be used to assess the accuracy of the various methods. In most methods reported in the literature, samples were purified before the measurement of ESTI. The principle of measurement was based on the ability of the samples to inhibit the sodium pump. These methods can be classified into the following types:

A. inhibition of purified Na,K-ATPase activity

B. inhibition of the sodium pumps in intact cells or tissues

C. biological effects of sodium pump inhibition on tissues, organs or animals, and

D. immunoreactivity with anti-digoxin or anti-ouabain antibodies.

PRETREATMENT AND PURIFICATION PROCEDURES PRIOR TO ESTI MEASUREMENT

Complexity of the pretreatment and purification procedures reported in the literature varied significantly depending on the purpose of the study. Research groups reporting the presence of ESTI in diseases have usually taken a simpler approach; while groups intending to purify the ESTI to homogeneity have developed more complicated procedures.

Simple pretreatment procedures have usually been carried out before the measurement or further purification steps. Some workers have incubated plasma samples at room temperature for 30 min to increase the measurable ESTI because it has been suggested that there are precursors (Gruber & Buckalew 1978, Pamnani & Haddy 1988). Others have boiled the samples for 5 - 20 min to deproteinise and increase the

yield of ESTI (Pamnani & Haddy 1988, Mattiasson & Öhlin 1987, Kelly *et al* 1985). Before boiling, the samples were diluted with water (Lau & Valdes 1988), acidified to pH 5.5 with hydrochloric acid (Hamlyn *et al* 1982, Balzan *et al* 1984), or both diluted and acidified (Boschi *et al* 1985, Bisordi & Holt 1989). Ultrafiltration has been another simple pretreatment procedure before or after deproteinization (Ng *et al* 1985, Gonick *et al* 1987, Weiler *et al* 1990). Solid phase extraction with reverse phase resins (e.g. C18, C8) to remove electrolytes and proteins has been one of the popular pretreatment methods. ESTI is adsorbed on the resin and then it was eluted with methanol (Devynck *et al* 1983, Vasdev *et al* 1985, Naomi *et al* 1991), different concentrations of ethanol (Boschi *et al* 1985, Buckalew *et al* 1987, Masugi *et al* 1987) or varying amount of acetonitrile in 0.1% trifluoroacetic acid (Morise *et al* 1986, Gonick *et al* 1987, Weber *et al* 1989, Weiler *et al* 1990). This popular technique also facilitates concentration of samples. Organic solvents are evaporated and the residues are reconstitued with a smaller volume of solvents than the starting sample volume. Lyophilization has also been commonly used to concentrate pretreated samples before purification (Balzan *et al* 1984, Boschi *et al* 1985, Bisordi & Holt 1989).

These methods of pretreatment could significantly affect the results. Moreth *et al* developed a quantitative receptor assay for the measurement of ESTI that did not require any pretreatment of the plasma samples (Moreth *et al* 1987). They were able to demonstrate that boiling of acidified plasma or ultrafiltration decreased the amount of ESTI in the samples. A significantly higher plasma ESTI concentration in the hypertensive patients could not be demonstrated after the samples were pretreated with these 2 methods. Boiling of acidified plasma for 10 min was reported to decrease ESTI by 25% and by 50% when boiling was prolonged to 20 min (Balzan *et al* 1984).

Procedures to purify biological samples for measuring or identifying ESTI have been mainly by chromatographic techniques (Kelly *et al* 1985, Goto *et al* 1989, Hamlyn *et al* 1989, Ludens *et al* 1991). Preparative column chromatography was initially used to handle the bulky samples. Further refinement would mainly depend on a

combination of high performance liquid chromatography (HPLC) methods such as ion-exchange chromatography, gel filtration and reverse-phase chromatography. Chromatographic eluants were collected to test for the presence of ESTI activity by different methods to be described in following sections. Separation by thin-layer chromatography has also been reported (Spustová *et al* 1985). Extraction methods using purified Na,K-ATPase preparations (Ludens *et al* 1991), anti-digoxin antibodies, affinity chromatography using anti-digoxin antibodies (Gruber *et al* 1983) and anti-ouabain antibodies (Kelly 1986) were used in an attempt to improve the specificity of the purified product. There is a common assumption that ESTI is a cardiac-glycoside like entity and the purification procedures should be optimized to recover substances of that nature (Hamlyn 1988, Schoner 1992, Goto *et al* 1992). However, this assumption had not been proven.

METHODS OF MEASURING ESTI

Inhibition of purified Na,K-ATPase activity

Purified Na,K-ATPase has been prepared from different sources. Common examples are dog kidney (Hamlyn *et al* 1982, Masugi *et al* 1987), hog cerebral cortex (Boschi *et al* 1985, Gonick *et al* 1987) and pig kidney (Moreth *et al* 1987). An exotic example was reported by Kelly *et al* who purified Na,K-ATPase from the supraorbital salt glands of ducks on high salt diet (Kelly *et al* 1985).

There has been a large variety of methods reported in the literature to measure the inhibition of Na,K-ATPase activity. The increase in the reaction product, phosphate, during the reaction was measured by standard chemical reactions (Roulston *et al* 1986, Masugi *et al* 1987). When radioactive adenosine triphosphate (ATP) was used as the substrate, the ^{32}P released was separated and counted on a gamma counter (Lau & Valdes 1988). The decrease in the substrate ATP was measured by a sensitive bioluminescence method and this facilitated the use of very low Na,K-ATPase activity to improve the sensitivity of the method (Weissberg *et al* 1984). An HPLC method has also been published to measure both ATP and ADP concentrations

simultaneously in the reaction mixture. The ratio of the two was used to calculate the Na,K-ATPase activity (Shimada *et al* 1985). The Na,K-ATPase reaction is coupled to pyruvate kinase and lactate dehydrogenase. The pyruvate kinase converts the product ADP back to ATP in the presence of phospho(enol)pyruvate producing pyruvate. Lactate dehydrogenase reduces pyruvate to lactate in the presence of reduced nicotinamide adenine dinucleotide (NADH). The rate of decrease in NADH concentration is monitored spectrophotometrically at 340 nm. Using this method, it was reported that ESTI in hypertensive patients was raised (Hamlyn *et al* 1982). A synthetic substrate, para-nitrophenylphosphate (PNPP), was successfully used to measure the inhibition of potassium-activated Na,K-ATPase activity in plasma from normotensive subjects with family history of hypertension (Weiler *et al* 1990). On the basis of competitive binding between radioactive [^{3}H]-ouabain and ESTI on the binding site of Na,K-ATPase, sensitive radioreceptor assays were developed (Moreth *et al* 1987, Hamlyn *et al* 1989). The percentage of tracer bound to Na,K-ATPase was inversely proportional to the concentration of the competing ESTI. The concentration of ESTI was usually expressed in ouabain-equivalent concentration.

The advantage of using this group of methods is mainly the ease of use. Purified Na,K-ATPase is available commercially and this obviates the necessity to purify the enzyme. Radioreceptor methods are sensitive and can measure ouabain in the nanomolar range. Furthermore, these methods can also be easily modified to distinguish ouabain-like and non-ouabain-like inhibition by varying potassium concentration (Masugi *et al* 1987). On the other hand, this group of methods suffers from many non-specific and specific interferences. Both intracellular and extracellular binding sites are available on the purified enzymes for inhibitor binding. However, only the extracellular binding sites are assessable *in vivo*. Several substances, including unsaturated fatty acids (Tamura *et al* 1985, Kelly *et al* 1986), lysophosphatidylcholine (Kelly *et al* 1986), ascorbic acid (Ng *et al* 1985, Kuske *et al* 1987), dehydroepiandrosterone sulfate (Vasdev *et al* 1985), and mammalian lignans (Braquet *et al* 1986), inhibit purified Na,K-ATPase, but are not able to inhibit sodium pumps on intact cells or tissues (Hamlyn 1988, Goto *et al* 1992). These substances do not demonstrate cardiac-glycoside activities. Nevertheless, lysophospholipids

were found to be natriuretic in rats (Rauch & Buckalew 1988). Therefore, the requirement that ESTI must inhibit the sodium pump similar to cardiac-glycosides has not yet been proven.

Anner *et al* reported the use of a novel two-sided liposome test system containing purified rabbit kidney Na,K-ATPase (Anner *et al* 1990). The active enzyme molecules are accessible on the exterior surface and the time required for inhibitors to reach the internal receptors can also be measured. This novel technique provides a system to overcome some of the disadvantages inherent to this group of methods. However, the preparation of such liposomes is very complicated and technically demanding.

Inhibition of the sodium pump on intact cells or tissues

A large variety of intact cells and tissues has been used to measure ESTI activities in biological fluids. Preparation of washed human red blood cells is a popular choice because it is easy to prepare and available in large quantity (Aronson *et al* 1977, Devynck *et al* 1983, Chimori *et al* 1986, Ludens *et al* 1991). Ghost red cell membranes after hypotonic shock have also been used (Kelly 1986, Buckalew *et al* 1987). Lymphocytes (Moreth *et al* 1986, Poston *et al* 1989) and platelets (Linder *et al* 1987, Mattiasson & Öhlin 1987) are the other common cells of human origin that have been used. They are also easily accessible but more tedious to work with. Although the use of human sigmoidal colon has been reported (Allgayer *et al* 1986), the difficulties in collecting enough human tissues limits its use.

As it has been suggested that ESTI could have *in vivo* effects on the renal tubules and blood vessels, intact cells and tissues were mostly chosen from these target organs (de Wardener & Clarkson 1985). The use of microtome sections of guinea pig kidney (Fenton *et al* 1982) and cultured renal tubular epithelial cell lines from canine and porcine (Goto *et al* 1988b) have been reported. On the blood vessel side, rat tail arteries (Pamnani *et al* 1988), rat thoracic aorta rings (Vasdev *et al* 1989), pig aorta (Stokes *et al* 1990), saphenous vein of dog's hind limb (Jandhyala & Ansari 1986), as well as tissue culture of rat aortic smooth muscle cells (Goto *et al* 1988a, 1989) have

been used. Other examples found in the literature include epithelia isolated from the frog (Cox & Woods 1987), fibroblast cell culture (Lichtstein *et al* 1985) and cortical slices of guinea pig brain (Ng *et al* 1985).

The principle of measurement in this group of methods is based on the functional aspects of the active sodium pump. The effects of ESTI on the ouabain-sensitive influx and efflux of sodium (Chimori *et al* 1986, Moreth *et al* 1986, Cox & Wood 1987, Poston *et al* 1989) and uptake of rubidium, an analogue of potassium (Aronson *et al* 1977, Ng *et al* 1985, Goto *et al* 1988b, Vasdev *et al* 1989, Stokes *et al* 1990, Ludens *et al* 1991) in different cells and tissues have been reported. Furthermore, inhibition of the sodium pump leads to an increase in intracellular calcium concentration. Therefore the movement of radioactive calcium into cultured cells has been used to assess the presence of ESTI in urine samples (Goto *et al* 1988a). Intracellular free calcium concentration in cells was also studied using fluorescent dye binding (Linder *et al* 1987, Goto *et al* 1989). The specific [^{3}H]-ouabain binding technique on intact cells has been another popular method used in this area of research (Devynck *et al* 1983, Allgayer *et al* 1986, Kelly 1986, Mattiasson & Öhlin 1987, Goto *et al* 1988b). The radioactive tracer was measured conveniently by standard counters. Using Scatchard analysis, the dissociation constant (K_D) and the amount of ESTI bound to each cell can be calculated.

de Wardener's group used a unique cytochemical technique to measure the inhibition of Na,K-ATPase activity in the proximal convoluted tubules of guinea pig kidney sections (Fenton *et al* 1982). Inhibition of Na,K-ATPase activity was shown to be correlated with the activation of glucose-6-phosphate dehydrogenase (G6PD) activity which had improved sensitivity over the inhibition of Na,K-ATPase activity. The Na,K-ATPase activity was measured by a hidden metal-capture principle (Chayen *et al* 1981). The G6PD activity was measured by the reduction of neotetrazolium salt to produce formazan deposits. The chromogens produced in these methods were quantitated by a scanning and integrating microdensitometer over a defined area of the tissue sections. It was necessary to dilute plasma samples 100 to 10000 fold. This is in contrast to other methods that require concentration of samples.

This group of methods was considered to offer the greatest sensitivity and utility in excluding most of the false positives, i.e. non-cardiac-glycoside-like inhibitors, detected by other methods (Hamlyn 1988). The choice of animals and tissues would be an important consideration because species-specific sensitivity of the tissues towards ouabain had been reported for partially purified ESTI from volume expanded plasma (Hamlyn *et al* 1988). The presence of 3 isoenzymes of Na,K-ATPase further complicate the issue as tissue-specific expression of enzyme activity has been reported (Goto *et al* 1992). Furthermore, the use of cells and tissues requires fresh sample preparation which is technically demanding. Biological variation among the animals is another obstacle. The use of specific tissue culture cell lines appeared to be a practical solution (Goto *et al* 1988a, 1988b, 1989). Finally, this group of measurement methods is still providing results based on *in vitro* conditions. The extrapolation of results to *in vivo* situations has still to be proven. Oral rubidium loading in humans has been used as an *in vivo* model to study cation transport in essential hypertension (Boon *et al* 1984). However, the results of this study do not support the presence of reduced Na,K-ATPase activity in untreated hypertensives.

Biological effects of sodium pump inhibition on tissues, organs or animals

Studies on volume-related humoral natriuretic factor have used methods which were based on the natriuretic effects of putative ESTI on animals or positive inotropic effects on tissues and organs. Examples of tissues and organs used were frog epithelia (Cox & Woods 1987), toad hemi-bladders (Granges *et al* 1986), rat tail arteries (Pamnani & Haddy 1988), guinea pig aorta and left atria (Bova *et al* 1991), rabbit femoral arteries and atria (Weber *et al* 1989) and human mesenteric and uterine arteries (Cappuccio *et al* 1986). The variety of animals used in this group of methods was relatively limited. Whole animals under anaesthesia such as dogs (Ahmad *et al* 1987) and rats (Buckalew *et al* 1987, Ebara *et al* 1988) have commonly been used. Restrained conscious rats have also been studied for detecting biological activity of partially purified urinary ESTI (Clarkson *et al* 1979).

The principle of measurement is based on the ability of ESTI to cause natriuresis and positive inotropic effects as a result of sodium pump inhibition. To monitor the effect on natriuresis, partially purified ESTI from different biological sources was infused into the whole animal via the tail vein (Clarkson *et al* 1979) or renal arteries (Ahmad *et al* 1987, Ebara *et al* 1988). Urine samples were collected over specified time periods. The urinary sodium concentration was measured by a standard laboratory method to calculate the output or fractional excretion. To monitor the positive inotropic effect, tissues were bathed in solutions containing varying amounts of extracted ESTI and a combination of electrodes was used to measure the short-circuit current, potential difference and direct-current resistance (Granges *et al* 1986, Cox & Woods 1987, Pamnani & Haddy 1988). Contraction response of tissues or organs as a result of positive inotropic effects was studied in the presence of the ESTI (Weber *et al* 1989, Bova *et al* 1991) or ESTI plus other vasoactive compounds such as norepinephrine and angiotensin II, that further sensitise the vasculature to the pressor effects (Cappuccio *et al* 1986, Weber *et al* 1989).

The biological effects on isolated organs and whole animals had been considered to be the classic method for the detection of ESTI. However, crude preparation of biological samples could contain interfering substances that also elicit the same biological response. For example, plasma atrial natriuretic peptide also causes natriuresis but not through binding to the sodium pumps (Ackermann 1986). Variations in the response of the tested animals could be due to the preparation of the animals. For example intrarenal infusion of ESTI preparation in denervated kidneys showed a higher natriuretic response than with intact kidneys (Bucklew & Gruber 1984). Acute stress, and even the use of different anaesthetics have been reported to cause abnormal ANF responses in rats (Horky *et al* 1985, Blizard & Morris 1987). A similar scenario for ESTI could lead to misleading conclusions.

Immunoreactivity with anti-digoxin and anti-ouabain antibodies

As ESTI is thought to mediate its biological action in a manner similar to cardiac glycosides, such as digoxin and ouabain, antibodies against cardiac glycosides are expected to crossreact with ESTI. The presence of digoxin-like immunoreactivity

(DLI) was described in humans, who were not on digoxin treatment, in a large variety of physiological and pathological conditions (Howarth *et al* 1990a and Goto *et al* 1991a). Thus, measurement of DLI has been used as a method to measure ESTI. On the other hand, studies on the use of ouabain-like immunoreactivity (OLI) to measure ESTI are comparatively scarce in the literature (Masugi *et al* 1986, 1987, Harris *et al* 1991). OLI was measured by polyclonal antibodies using an ELISA method (Harris *et al* 1991). Measurement of DLI was mainly performed with commercially available reagent kits using techniques like solid phase radioimmunoassay (Balzan *et al* 1984), double antibody radioimmunoassay (Gruber *et al* 1983), and automated fluorescence polarisation immunoassay (Howarth *et al* 1990b). In one study, 7 different commercial digoxin kits were employed to develop the "immunochemical fingerprint" for serum samples collected from different clinical conditions as well as several proposed ESTI (Naomi *et al* 1991). This approach clearly demonstrated the differences between the cross reactivity patterns for different preparations of anti-digoxin antibodies. Most research groups attempted to modify the manufacturers' recommended protocol for measurement. Attempts to lower the detection limit and to add lower standards were common practice to cover the ESTI concentration that was usually on the lower side of the digoxin standard curve. The detection limit was decreased, for example, by increasing the sample to tracer volume ratio (Wijdicks *et al* 1987).

The use of immunoreactivity to measure ESTI is convenient and this method is available in many clinical laboratories, mainly using commercial digoxin kits. The affinities of these antibodies vary for ESTI. Other interfering substances, for example, unsaturated fatty acids (Young *et al* 1986) and bile salts (Vasdev *et al* 1986) can cause interference. Cross-reactivity studies with steroids show that some steroids cause measurable DLI only at supra-physiological concentrations (Wong *et al* 1992).

It was suggested that ESTI might share the same ligand recognised by the anti-digoxin antibodies (Gruber *et al* 1980). Goto *et al* argued against the use of DLI to measure ESTI because the recognition sites of anti-digoxin antibodies are different from that of the cardiac glycoside receptor (Goto *et al* 1992). However, it has been

reported that affinity purification using highly specific anti-digoxin antibody reduced the binding of partially purified urinary ESTI to cardiac glycoside receptors (Goto *et al* 1991b). This result showed that anti-digoxin antibodies competed with the cardiac glycoside binding sites for ESTI. In another *in vivo* study, infusion of high affinity digoxin-binding Fab fragments into rats removed ESTI from erythrocytes and kidney cortex (Wagener *et al* 1989). On the other hand, there have been studies showing that DLI did not correlate with other methods of measuring ESTI (Hamlyn *et al* 1982, Clarkson & de Wardener 1985, Yamada *et al* 1988, 1990, Wong *et al* 1992).

The recent identification of ouabain as one of the plasma ESTI (Ludens *et al* 1991) provides a strong case for the use of OLI measurement. However, there is only limited information available in the literature on the performance of this method. Non-specific interference from lipids or steroids would be similar to that reported for anti-digoxin antibodies.

Concluding remarks

There has been a large variety of methods for the measurement of ESTI reported in the literature. However, as the nature of ESTI has not been clarified, it is difficult to decide which method of measuring ESTI is superior to others. Although there have been strong views that ESTI functions like cardiac-glycosides, there are others with conflicting views. The choice of method for the measurement of ESTI would affect the outcome of the study. More than one method should be used to characterise the ESTI activity in any study. It is generally agreed that it is possible to have more than one compound that can promote natriuresis under different physiological and pathophysiological conditions.

II. METHOD OF MEASUREMENT OF ESTI IN THIS STUDY

PRINCIPLES OF METHODS

Two methods were developed for the measurement of ESTI in this project, without assuming the nature of ESTI. The first method was a sensitive and convenient technique to measure the inhibition of purified Na,K-ATPase activity. A pretreatment step was required to remove interferences and maximise the inhibitory activity in plasma and urine samples. The second method was to measure DLI using radioimmunoassay (RIA). During the later part of the project, the production of the RIA reagent kit was discontinued by the manufacturer and an automated homogenous enzyme immunoassay (HEIA) was used for the same purpose.

Inhibition of purified dog kidney Na,K-ATPase activity

ESTI is extracted by Sep Pak C18 resin. Proteins and electrolytes are removed during this pretreatment step to eliminate their interference on the subsequent reactions. In the presence of sodium and ATP, extracted ESTI is allowed to bind onto and inhibit a purified dog kidney Na,K-ATPase preparation in a potassium free medium. The residual ATPase activity is measured on an automated analyser by an enzyme-coupled reaction as illustrated by the following reactions:

$$\text{ATP} \xrightarrow{\text{Na,K-ATPase}} \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{PYRUVATE} + \text{ATP}$$

$$\text{PYRUVATE} + \text{NADH} \xrightarrow{\text{LDH}} \text{LACTATE} + \text{NAD}$$

The activity is determined by the rate of change of NADH concentration monitored at 340 nm. The percentage of inhibition (%I) of the extract is compared with the %I of

a series of aqueous ouabain standards and the concentration of ESTI is expressed as %I or as nmol of ouabain equivalent (OE).

In this method, the hydrolysis of ATP is linked to a regenerating system in which pyruvate kinase (PK) catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate with the consequent reconversion of the ADP to ATP. This maintains the concentration of ATP constant and prevents the accumulation of ADP, which is an ATPase inhibitor. The incorporation of a 2-h incubation of the extracted ESTI with the enzyme preparation improved the sensitivity of the method. Another advantage of this method is the possibility of automation using routine clinical chemistry analysers. The use of a centrifugal analyser in this project facilitated the measurement of 28 samples in less than 20 minutes.

Measurement of DLI by radioimmunoassay

The RIANEN radioimmunoassay has been a popular method for the measurement of DLI (Vasdev *et al* 1985, Lau & Valdes 1988, Bisordi & Holt 1989, Naomi *et al* 1991). Standards or samples and a fixed amount of the [^{125}I]-labelled digoxin are allowed to react with a constant and limiting amount of anti-digoxin antibody. The amount of the labelled antigen bound to the antibody is inversely proportional to the amount of unlabelled antigen. In this particular kit, separation of the bound from free antigen is achieved by a pre-reacted first and second antibody complex. With such a system, the pipetting and reaction times are reduced compared to the standard sequential double antibody assay. After a single 30 min incubation followed by centrifugation, the supernatant is discarded and the pellet is counted to quantitate the bound tracer. The normalised percent bound (%B/ B_0) data are used to construct a standard curve from which the values of the unknowns are obtained by interpolation.

The manufacturer supplied the typical cross-reactivities of the antibody. The reported percentages indicate cross reactivity at 50% displacement compared to digoxin. Cross-reactivities of the different compounds tested were: digitoxin 5.3%; acetyldigitoxin 1.1%; progesterone 0.056%; ouabain, prednisolone and cortisol <0.02%; testosterone, corticosterone, spironolactone, desoxycortisol and cortisone

<0.01%; 17α-estradiol, 17β-estradiol, pregnenolone, prednisone and cholesterol <0.0002%.

Measurement of DLI by HEIA

The CEDIA Digoxin kit supplied by Microgenic was chosen to replace the RIA. This HEIA method has the advantages that it can be easily automated on a large variety of clinical chemistry analysers and requires no sample pretreatment. The method employs 2 inactive polypeptide fragments of β-galactosidase (EC 3.2.1.23) produced by recombinant DNA technology (Henderson *et al* 1986). One fragment, called the enzyme-acceptor (EA), is a polypeptide with a small sequence missing in the encoded primary structure. The other fragment, called the enzyme-donor (ED), contains the missing sequence of the EA polypeptide. EA and ED can reassociate spontaneously in solution to form a tetrameric enzymatic entity as active as the native β-galactosidase. In this kit, digoxin is conjugated to the ED that competes with digoxin in the sample for binding sites on a digoxin-specific antibody. Due to steric hindrance, the digoxin-ED conjugate bound to the digoxin antibody is prevented from reassociation with EA to form an active enzymatic entity. After the competitive binding, hydrolysis of the substrate o-nitrophenyl-β-galactopyranoside, monitored at 420 nm, is used to measure the residual enzyme activity that is directly proportional to the concentration of digoxin in the sample.

According to the manufacturer, the drugs that show clinically significant interference with this method include digitoxigenin, digitoxin, digoxigenin and deslanoside. Cross-reactivities of the different compounds tested were:: ouabain 2.9%, gitalin 29.0%, and less than 0.1% for furosemide, prednisolone, prednisone, quinidine, spironolactone, testosterone, and theophylline.

MATERIALS AND METHODS

Materials

Sodium chloride (NaCl); potassium chloride (KCl); magnesium sulphate($MgSO_4$); TES and TRIS buffers; ouabain; phosphoenolphosphate (PEP); nicotinamide adenine dinucleotide reduced form (NADH); adenosine triphosphate disodium salt, grade I (ATP); ethylenediaminetetraacetic acid (EDTA); ethylene glycol Bis (ß-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA); sodium potassium activated, ouabain sensitive, adenosine triphosphatase from dog kidney, grade IV (Na,K-ATPase); pyruvate kinase (PK) and lactate dehydrogenase (LD) from rabbit muscle; were purchased from Sigma Chemical Company (St. Louis, MO, USA). Water was subjected to reversed osmosis and deionization by the Milli-Q water purification system (Millipore, Milford, MA, USA). Methanol and concentrated hydrochloric acid (HCl AR grade) were obtained from Merck (Darmstadt, Germany). C18 Sep Pak cartridges were from Waters Company (Millipore, Milford, MA, USA).

The RIA digoxin kit, RIANEN™, was from New England Nuclear (North Billerica, MA, USA). The HEIA digoxin kit was from Microgenic (Concord, CA, USA). Theree levels of TDX digoxin quality control samples were from Abbott Laboratories (North Chicago, IL, USA).

Equipment

The SpeedVac™, a centrifuge connected to a vacuum pump for rapid evaporation of volatile solvent, was from Savant Instruments (Farmingdale, NY, USA). The Cobas Bio™ and Cobas Fara™, centrifugal chemistry analysers, were from Hoffman La Roche (Basle, Switzerland). The gamma counter was from Packard Instruments (Downers, IL, USA).

Reagent preparation

Enzyme suspension solution

This is a 10 mmol/l TES/TRIS buffer solution containing 0.5 mmol/l of EDTA (free acid), pH 7.4 at 37 °C and is stable at 4°C for at least 6 months. EDTA (0.01462 g),

TRIS (0.1211 g) and TES (0.2292 g) were dissolved in 80 ml of deionized distilled water. The pH was adjusted to 7.4 at 37 °C with 1 M HCl. The solution was made up to volume in a 100-ml volumetric flask.

Stock Na,K-ATPase solution

A 5-Unit bottle of lyophilized Na,K-ATPase was reconstituted with 1 ml of enzyme suspension solution and incubated at room temperature for 3 hours. The solution was then aliquoted into small Eppendorf centrifuge tubes (50 µl in each tube) and kept frozen at -70 °C. This stock solution is stable for at least 6 months.

Buffer A

This is a 50 mmol/l of TRIS buffer solution containing 100 mmol/l of NaCl, 4 mmol/l of $MgSO_4$ and 0.5 mmol/l of EDTA (free acid), pH 7.4 at 37 °C. It is stable for at least 6 months when stored at 4 °C. TRIS (6.055 g), NaCl (5.844 g), $MgSO_4$ (0.4816 g) and EDTA (0.1462 g) were dissolved in 800 ml of deionized distilled water. The pH was adjusted to 7.4 at 37 °C with diluted HCl solution. The buffer was made up to volume in a 1-litre volumetric flask.

Na,K-ATPase working solution (Reagent D)

The working solution was prepared by a 1 in 20 dilution of the stock enzyme solution with the Buffer A immediately before use. This solution is stable for 12 hours at 4 °C. The resulting Na,K-ATPase activity would be in the range of 200 - 250 U/l depending on the lot of the stock enzyme.

Ouabain standard solutions

Working ouabain standard solutions of 5, 10, 20, 30, and 50 nM were prepared by appropriate dilution of an aqueous 1 µM ouabain stock solution with the Buffer A in volumetric flasks.

ATP solution for incubation (Reagent F)

This is a 5mmol ATP in 5 ml of Buffer A solution and is stable for at least 12 hours when kept at 4 °C. ATP (0.0152 g) was dissolved in 5 ml Buffer A fresh prior to use.

Buffer B

This is a 112 mmol/l of TES/TRIS buffer solution containing 200 mmol/l of NaCl, 56 mmol/l of KCl, 13.6 mmol/l of $MgSO_4$, and 14 mmol/l of EGTA, pH 7.4 at 37 °C. This solution is stable for at least 6 months when kept at 4 °C. TRIS (13.56 g), TES (25.67 g), NaCl (11.69 g), $MgSO_4$ (4.173 g) and EGTA (5.318 g) were dissolved in 800 ml of deionized distilled water. The pH was adjusted to 7.4 at 37 °C with 1 M HCl. The buffer was made up to volume in a 1-litre volumetric flask.

ATPase assay reagent (Reagent H)

This reagent solution contains 7.4 mmol/l of ATP, 3.4 mmol/l of PEP, 1.5 mmol/l of NADH, 3400 U/l of PK, and 4800 U/l of LD in Buffer B. This reagent is stable for at least 12 hours when kept at 4 °C . ATP (0.0449 g), PEP (0.0170 g), NADH (0.0112 g) and 55 µl of PK/LD solution were dissolved in 10 ml of Buffer B, prepared fresh prior to use.

Methods

Extraction of plasma ESTI using Sep Pak cartridges

A. A Sep Pak C18 cartridge was primed with 2 ml of methanol and washed with 10 ml of deionized distilled water.

B. 300 µl lithium heparin plasma sample was carefully applied into the Sep Pak cartridge. After the plasma was all inside the compartment holding the resin, it was allowed to stay inside the compartment for 5 min.

C. The Sep Pak cartridge was washed with 20 ml of deionized distilled water to remove protein and electrolytes under vacuum. Water trapped inside the Sep Pak was removed as much as possible.

D. ESTI was eluted slowly from the C18 resin with 2 volumes of 1-ml methanol.

E. The methanol extract was separated into 3 equal portions and each portion was kept in an Eppendorf microcentrifuge tube.

F. The methanol was evaporated to dryness (either by blow-drying under nitrogen or in the Speed-Vac). The methanol extract could be stored overnight at -70 °C before the evaporation and the dry residue could be stored at -70 °C for at least a month before analysis.

G. Each plasma sample was extracted twice and the mean of the duplicates is presented.

Extraction of urine ESTI using Sep Pak cartridges

The extraction of urine ESTI by Sep Pak cartridges was similar to the procedure described for the plasma samples. Due to the large variation of urinary concentration of ESTI, it was necessary to concentrate the urine sample, usually up to 3 times the original concentration.

A. 300 µl of urine was carefully applied to the Sep Pak cartridge and the sample was allowed to stay inside the resin compartment for 2 min. The cartridge was washed with 20 ml of deionized distilled water under vaccum.

B. The above procedure was repeated twice with further 300 µl of the urine sample.

C. Then, urine ESTI from the 900 µl of urine sample was eluted with two volumes of 1-ml methanol.

D. The subsequent procedure was as described for the extraction of plasma samples.

E. For each urine sample, extraction was done with 300 µl, 600 µl, and 900 µl of sample and this produced 1 fold, 2 fold and 3 fold concentrated extract.

Incubation with Na,K-ATPase

A. One portion of the dry residue was reconstituted in the Eppendorf tube with 100 µl of Buffer A. To ensure complete redissolution of the extracted ESTI into the buffer solution, the solution was vortexed regularly for at least 1 hour.

B. 100 µl of each ouabain standard solution was pipetted into different Eppendorf tubes and Buffer A was used as the zero standard in duplicates.

C. All tubes were kept in an ice bath while Reagent D and Reagent F were prepared.

D. 50 µl of reagent D and 50 µl of reagent F were added to each Eppendorf tube. The tubes were capped and vortex-mixed. The capped tubes were still kept in an ice bath until all the tubes were prepared.

E. All the tubes were incubated in a 37 °C water bath for 2 hours. They were then transferred to an ice bath to stop the binding of ESTI to the Na,K-ATPase.

F. The tubes were kept in the ice bath until the residual activity was measured on the Cobas Bio.

Measurement of ATPase activity using a centrifugal analyser

A. Reagent H was prepared and placed in the main reagent compartment of the reagent container.

B. The content of each Eppendorf tube was transferred to a Cobas Bio sample tube that was also kept in ice bath and then transferred to the sample ring.

C. A new cuvette rotor was always used for the measurement.

D. The Cobas Bio analyser was programmed as shown on Table 2-1.

E. After the reaction was completed, the residual Na,K-ATPase activity was calculated, and the %I for each sample was calculated by comparing with the mean enzyme activity of the duplicate zero tubes.

F. A standard curve was plotted and %I of each sample was compared with the standard curve to determine the plasma ESTI concentration manually, in ouabain equivalent unit (OE) either manually or using a non-linear curve fitting program on a personal computer.

Table 2-1: Cobas Bio Programming for the measurement of Na,K-ATPase activity

	α	13 (-)
1.	Units	U/l
2.	Calculation factor	4013
3.	Standard 1 Conc	0
4.	Standard 2 Conc	0
5.	Standard 3 Conc	0
6.	Limit	0
7.	Temperature (°C)	37.0
8.	Type of analysis	2
9.	Wavelength (nm)	340
10.	Sample volume (µl)	40
11.	Diluent volume (µl)	50
12.	Reagent volume (µl)	50
13.	Incubation time (sec)	0
14.	Start reagent volume (µl)	0
15.	Time of first reading (sec)	300.0
16.	Time interval (sec)	30
17.	Number of readings	21
18.	Blanking mode	0
19.	Printout mode	1

Measurement of DLI by radioimmunoassay

The preparation of reagents and the procedure for the measurement of DLI were according to the manufacturer's instruction except that the amount of sample and reagent volumes were reduced proportionally 2.5 times.

A. Samples were extracted using Sep Pak cartridges as described in the previous section. Residues in the Eppendorf tubes were reconstituted with 100 µl of zero standard solution supplied by the manufacturer and left to stand for at least 1 h at room temperature with intermittent vortexing. 40 µl of standards, quality control samples and sample extracts were pipetted into plastic tubes in duplicates.

B. 200 µl of the blue tracer solution was added to each tube.

C. Then, 200 µl of the digoxin antiserum complex was added to each tube.

D. Each tube was vortexed for 5 - 10 sec and the tubes were incubated at room temperature for 30 min.

E. All the tubes were centrifuged at 1000 g for 10 min in a 4°C refrigerated centrifuge.

F. The supernatants were aspirated and the antibody complex in each tube was counted on a gamma counter for 1 min.

G. Digoxin concentration were calculated by automatically using the program in the counter.

Measurement of DLI by HEIA

The preparation of reagent was according to the manufacturer's recommendation. However, the reagent and sample volumes were reduced proportionally by about 50% to increase the number of tests per kit.

A. Samples were extracted using Sep Pak cartridges as described in the previous section. Residues in the Eppendorf tubes were reconstituted with 100 µl of zero standard solution supplied by the manufacturer andl left to stand for at least 1 h at room temperature with intermittent mixing. The reconstituted samples were then transferred to the Roche sample tubes and placed on the sample ring. Three levels of digoxin quality control samples were also included in each batch of analysis.

B. On the reagent container, the 3 standards were placed in the standard positions, the enzyme donor/antibody reagent was placed in the main reagent compartment, and the enzyme acceptor / substrate was placed in the start reagent compartment.

C. The Cobas Fara was programmed as shown on Table 2-2.

Table 2-2: Cobas Fara programming for the measurement of DLI using the CEDIA reagent kit.

GENERAL

MEASUREMENT MODE :	ABSORB
REACTION MODE :	CEDIA
CALIBRATION MODE :	STD NONLIN
REAGENT BLANK :	NO BLANK
WAVELENGTH :	420 nm
TEMPERATURE :	37.0 °C
DECIMAL POSITION :	2
UNIT :	µg/l

ANALYSIS

P	SAMPLE :	15µl	DILUENT :	6µl
	REAGENT :	75µl		
T	TEMP. DELAY :	0 s		
I0	INCUBATION :	120 s		
SR1	START R1 :	45µl	DILUENT :	12µl
I0	INCUBATION :	700S		
A0	READINS :	0.5 s	NUMBER :	21
	INTERVAL :	10 s		

CALCULATION

CONVERS. FACTOR :	1.0000	OFF :	0.0000
REAC DIRECTION :	INCREASE	CHECK :	ON
SAMPLE LIMIT :	NO		
TEST RANGE :	NO	HI :	NO
NORM RANGE :	NO	HI :	NO
CALC STEPS :	1		
CALC STEPS A:	KINETIC		
READINGS FIRST :	2	LAST :	21
REAC. LIMIT :	NO		

CALIBRATION

CALIB. INTERVAL :	EACH RUN
STANDARD NONLINEAR :	
1: 0.00	2: 2.00
3: 4.00	
DEVIATION :	NO
CALC MODEL :	LINREGRESS
REPLICATE :	SINGLE
CORRECTION STD :	OFF

REACTION MODE :

NAME	CEDIA	
1.	P	PARALLEL PIPETTING
2.	T	INCUBATION SPEC
3.	I0	INCUBATION
4.	SR1	START REAGENT 1
5.	I0	INCUBATION
6.	A0	MEASUREMENT CYCLE

RESULTS

Optimization of reagent composition for the measurement of Na,K-ATPase activity

Optimization of the reagent composition for the measurement of Na,K-ATPase activity was performed on the Cobas Bio. The programming of the centrifugal analyser was similar to that described on Table 2-1.

Optimization of sodium concentration

A series of NaCl solutions was prepared by appropriate dilution of a stock NaCl solution in TES/TRIS buffer. Six different Reagent H solutions were prepared from these NaCl solutions so that the final reaction mixture contained the following sodium concentrations: 50, 75, 100, 125, 150 and 175 mmol/l. These solutions were added to the main reagent compartments of six different reagent trays. The Reagent F (Na,K-ATPase working solution) was modified to contain an activity of about 100 U/l and it was placed into 3 different sample tubes on the sample ring. When the analyser completed the pipetting sequence for the first Reagent H solution, the system was interrupted and the second Reagent H solution was introduced to replace the first one. The interruption continued until all the different Reagent H solutions were pipetted into the cuvette rotor. After the reaction was completed, mean value of the measured Na,K-ATPase activity were plotted against the final sodium concentrations.

Changes in Na,K-ATPase activity with increasing sodium concentration is shown in Figure 2-1 (A). There was a significant increase in enzyme activity between 0 and 50 mmol/l of sodium. The enzyme activity peaked at 100 mmol/l of sodium and further increases of sodium concentration led to a gradual decrease in activity. Therefore, an optimal concentration of 100 mmol/l sodium was selected.

Optimization of potassium concentration

The procedure was similar to that described for the optimization of sodium. KCl solutions were used to provide a final K concentration of 0, 5, 10, 15, 20, 25 and 30 mmol/l. As shown in Figure 2-1 (B), Na,K-ATPase activity increased by more than 60% when the potassium concentration increased from 0 to 10 mmol/l. However, the

enzyme activity was relatively unchanged with higher potassium concentrations. A final concentration of 20 mmol/l potassium was selected.

Optimization of ATP concentration

The procedure was similar to that described for the optimization of sodium. ATP solutions were used to provide a final concentration of 0, 1, 2, 3, 4 and 5 mmol/l. The results are shown in Figure 2-1 (C). As expected, there was no measurable Na,K-ATPase activity in the absence of ATP. The activity increased sharply and reached the highest activity at 3 mmol/l. Further increase of the ATP concentration, however, gradually decreased the activity. A final concentration of 3 mmol/l ATP was selected.

Optimization of magnesium concentration

The procedure was similar to that described for the optimization of sodium. $MgSO_4$ solutions were used to provide a final concentration of 0, 2, 4, 6, 8 and 10 mmol/l. The results are shown in Figure 2-1 (D). Na,K-ATPase activity was not measurable in the absence of magnesium. The activity sharply rose with increasing magneisum concentrations with an optimal concentration at 6 mmol/l. Further increase of the magnesium concentration had no significant effect on the Na,K-ATPase activity. A final concentration of 6 mmol/l magnesium was selected.

Optimization of EGTA concentration

The procedure was similar to that described for the optimization of sodium. EGTA solutions were used to provide a final concentration of 0, 2, 4, 6, 8 and 10 mmol/l. The results are shown in Figure 2-1. Optimal Na,K-ATPase activity was obtained at 2 mmol/l concentration and further increase of EGTA concentrations inhibited the enzyme in a dose response manner. A final concentration of 2 mmol/l EGTA was selected.

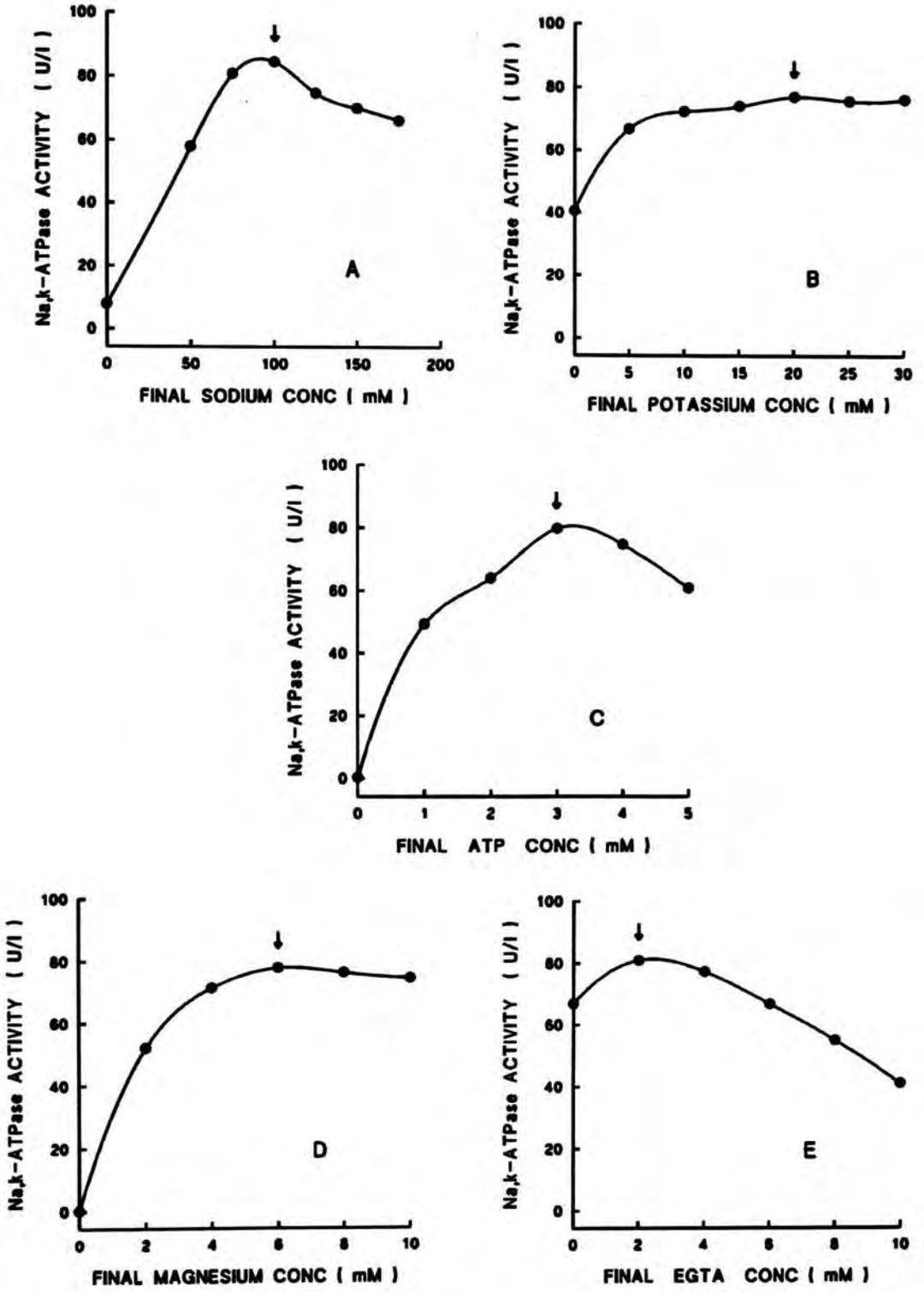


Figure 2-1: **Effects of different concentrations of sodium (A), potassium (B), ATP (C), magnesium (D) and EGTA (E) on Na,K-ATPase activity. The optimal concentrations are marked with an arrow.**

Effect of sample pretreatment on the recovery of solid phase extraction

In order to improve the efficiency of extracting ESTI, numerous methods of sample pretreatment before Sep Pak extraction were evaluated.

Sample dilution, acidification, and treatment with organic solvents

A pool of cord plasma was collected from the samples sent to the routine laboratory for neonatal hypothyroid screening. Another pool of lithium heparin plasma was collected from healthy adult volunteers. Both pools of plasma samples were subjected to the following pretreatment methods in triplicates:

A.	No treatment
B1.	Dilution with one volume of deionized distilled water
B2.	Dilution with 2 volumes of deionized distilled water
B3.	Dilution with 4 volumes of deionized distilled water
C1.	Same as B1, pH adjusted to 5.5 with 1 M HCl
C2.	Same as B2, pH adjusted to 5.5 with 1 M HCl
C3.	Same as B3, pH adjusted to 5.5 with 1 M HCl
D.	Dilution with 9 volumes of methanol/acetone (1:1,v/v)
E.	Dilution with same volume of 30% Trifluoroacetic acid (TFA)
F.	Dilution with 9 volumes of 3% of Trichloroacetic acid (TCA)

After the pretreatment, the sample tubes were centrifuged at 1600 g for 10 min to remove protein precipitates. The supernantants were extracted using Sep Pak cartridges as described in the METHOD section except that no equilibration time was introduced. The supernatants were allowed to pass through the cartridges slowly under controlled vaccum. The ESTI was eluted with methanol, dried, reconstituted, and analysed as described in the METHOD section. Incubation with Na,K-ATPase was done on the Cobas Bio for 5 min before the measurement of residual activity. The mean %I for each method was calculated. The result for method A was taken as 100% and all other results were expressed in relation to this.

The results for both pools of plasma samples are tabulated on Table 2-3. The different pretreatment methods did not significantly improve the recovery of ESTI from cord plasma or healthy adult plama. Dilution of the sample with deionized

distilled water increased the recovery slightly for healthy adult plasma but had no effect on cord plasma. The acidification of both types of samples to pH 5.5 gave lower %I compared to no pretreatment. The use of organic solvents or strong acids for pretreatment gave even lower recovery.

Boiling as sample pretreatment method before Sep Pak extraction

To study the effect of boiling on the recovery of ESTI, a cord plasma pool was used. Five ml of cord plasma was added to a 10-ml glass test tube and placed into a boiling water bath for 1 min in duplicates. The cord plasma proteins coagulated into gelatinous form. A glass rod was used to disrupt the gel thoroughly before the tubes were centrifuged at 50000 g for 10 min. Two ml of the supernatant was passed through the Sep Pak for extraction of ESTI. The other procedures were similar to those described in previous section. The procedure was then repeated for boiling times of 2, 4, 5, 10 and 20 min.

The results are shown in Table 2-4a. A short boiling time of 1 min gave similar %I results as the unboiled sample. However, longer boiling duration reduced the %I . A boiling time of more than 5 min abolished the inhibitory ability of the cord plasma.

Another experiment was similarly set up to study the effect of sample volume upon boiling of the cord plasma sample for 5 min. The different sample volumes used were: 5, 10, 15 and 20 ml. Results are shown in Table 2-4b. The larger sample volumes preserved the inhibitory ability better, probably due to the uneven effect of heating for a short period of time.

Results of the above experiments indicated that the uses of these pretreatment methods before Sep Pak extraction of ESTI in adult plasma or cord plasma samples did not improve the recovery. On the contrary, some of the pretreatment methods lowered the recovery dramatically. Since the nature of the ESTI was not known, the method that gave the highest inhibition would be considered desirable. None of the above pretreatment was considered further.

Table 2-3: **The effect of different sample pretreatment methods on the recovery of ESTI on solid phase extraction using Sep Pak cartridges.**

Methods	Normalized Mean %I	
	Cord plasma	Healthy adult plasma
A	100	100
B1	91	78
B2	78	106
B3	76	115
C1	84	92
C2	86	74
C3	81	70
D	44	79
E	0	0
F	18	0

Methods :

A.	No treatment
B1.	Dilution with same volume of deionized distilled water
B2.	Dilution with 2 volumes of deionized distilled water
B3.	Dilution with 4 volumes of deionized distilled water
C1.	Same as B1, adjust pH to 5.5 with 1 M HCl
C2.	Same as B2, adjust pH to 5.5 with 1 M HCl
C3.	Same as B3, adjust pH to 5.5 with 1 M HCl
D.	Dilution with 9 volumes of methanol/acetone (1:1,v/v)
E.	Dilution with same volume of 30% Trifluoroacetic acid (TFA)
F.	Dilution with 9 volumes of 3% of Trichloroacetic acid (TCA)

Pooled cord plasma and lithium heparin plasma samples were pretreated with the above methods. The samples were then extracted for ESTI using Sep Pak. The % inhibition measured for method A was converted to 100% and the %Inhibition of other methods were then normalized to method A.

Table 2-4a: Effects of boiling for different times on the recovery of cord plasma ESTI by Sep Pak extraction.

Boiling time (min)	Normalized Mean %I
0	100
1	107
2	65
4	43
5	7
10	0
20	0

The %I for the unboiled sample was taken as 100% and the results of other boiling times were normalized to the unboiled sample.

Table 2-4b: Effects of sample volume upon boiling for 5 min on the recovery of ESTI by Sep Pak extraction.

Sample volume (ml)	Mean % I
5	12
10	33
15	39
20	50

Modification of the Sep Pak extraction method

Lipids are believed to be non-ouabain-like inhibitors of the sodium pump and interfere with the measurement of the ouabain-like ESTI. Non-polar organic solvents were used to wash the Sep Pak C18 resin before the elution of ESTI with methanol. This modification was intended to remove lipids which coelute with ESTI, to improve the specificity of the method.

Lithium heparin plasma sample was collected from a healthy volunteer and 1 ml of the plasma sample was loaded onto the Sep Pak cartridge as described in the previous sections. Washing of the cartridge with deionized distilled water and elution of ESTI with methanol were as described in the METHOD section. Incubation of the extracted ESTI with Na,K-ATPase for 30 sec was performed on the Cobas Bio. The measurement of residual Na,K-ATPase activity was as described in the METHOD section. Samples were analysed in triplicate.

The above procedure was then repeated with the following additional washing step before the methanol elution:

A. 5 ml ether
B. 5 ml ether + 5 ml acetone
C. 5 ml ether + 5 ml acetonitrile
D. 5 ml ether + 5 ml chloroform
E. 5 ml ether + 5 ml chloroform + 5 ml acetone
F. 5 ml ether + 5 ml chloroform + 5 ml acetonitrile

The eluted organic solvents were each collected into individual glass test tubes. The solvents were evaporated to dryness in a vacuum oven at 40 °C. The residues were reconstituted with 500 µl of Buffer A. ESTI was measured in these fractions.

There was no measurable ESTI in any of the ether, acetone or acetonitrile fractions. In the chloroform fractions, %I ranged from 1.0 to 7.7% with very poor precision for the triplicates. It was later learned from the manufacturer of the Sep Pak cartridges that chloroform has some intrinsic properties that damage the C18 resin and it should not be used for elution with the C18 cartridges. Thus, results of modifications D, E and F were discarded. Plasma ESTI was detected in all methanol fractions for

modifications A to C. %I of these 3 methanol fractions was about 50% lower than the methanol fraction without any modification. However, the precision of the triplicates was very poor. The effects of combining the different organic solvents on the functioning of the C18 resin with the ESTI were not known. The observed poor precision could be a result of the complicated interactions among the multiple components involved. Thus, modification of the Sep Pak procedure using these organic solvents was not continued further.

Evaluation of the efficiency of the Sep Pak cartridges

The Sep Pak cartridges were recommended to be used only once by the manufacturer. However, the C18 resins could be regenerated by washing with methanol and water. The regeneration process can reduce the running cost of measuring ESTI. The cord plasma pool used to study the effect of sample pretreatment was used in this experiment to evaluate the number of times that a Sep Pak cartridge can be used for effective extraction of ESTI. Cord plasma was chosen instead of adult plasma because of the higher ESTI present in the cord plasma sample.

Eight Sep Pak cartridges were prepared for the extraction of cord plasma ESTI as previously described. After the methanol elution step, the Sep Pak cartridges were regenerated with 5 ml of methanol wash, followed by 10 ml of deionized distilled water. Each Sep Pak cartridge was used 12 times. Extracts were incubated with Na,K-ATPase for 30 sec on the Cobas Bio before the measurement of enzyme activity as described previously. Statistical analyses of the %I were performed by the WinSTAR™ Statistic program (Anderson Bell, CO, USA). Analysis of variance was used to look for significant differences between the number of times of extraction.

The results are shown on Table 2-5. The %I of the first 6 batches of extraction were not statistically different from one another. The mean %I values decreased steadily from the 7^{th} round onwards and were all statistically different from the first set of results. The precision performance also deteriorated after 6 rounds of extraction. The Sep Pak cartridges were all used for not more than 5 extractions for the rest of the study.

Table 2-5: **Efficiency of Sep Pak cartridges for the extraction of cord plasma ESTI. Eight Sep Pak cartridges were used for 12 successive extractions.**

No. of extraction	Mean %I (n=8)	Coefficient of variation (%)
1	62.4	7.1
2	64.4	6.7
3	67.5	3.7
4	64.7	3.4
5	61.4	4.6
6	62.0	5.2
7	49.9	10.8
8	39.3	24.7
9	47.8	11.5
10	43.7	10.1
11	41.9	3.6
12	32.5	24.2

Effect of equilibration time between plasma ESTI and the C18 resin on the recovery and precision of the extraction step

This experiment was designed to improve the precision of the extraction of plasma ESTI. A plasma sample was collected from a healthy male volunteer on the 7th day of a high salt diet. 250 µl of the plasma sample was allowed to be in contact with the C18 resin inside the Sep Pak cartridge for 10 seconds before the subsequent washing and extraction steps. Methanol extracts were evaporated and analysed as described in the METHOD section. The %I of each extraction was calculated. These processes were repeated with 2 other new Sep Pak cartridges.

The above procedure was then repeated except that the sample was allowed to stay inside the Sep Pak cartridges for 1, 2, 3, 5, 10 and 15 min.

Results of this experiment are shown in Figure 2-2. The short equilibration time of the plasma with the C18 resin resulted in poor precision and lower recovery of ESTI. An equilibration time of 5 min was chosen as further increase in time did not improve the performance significantly.

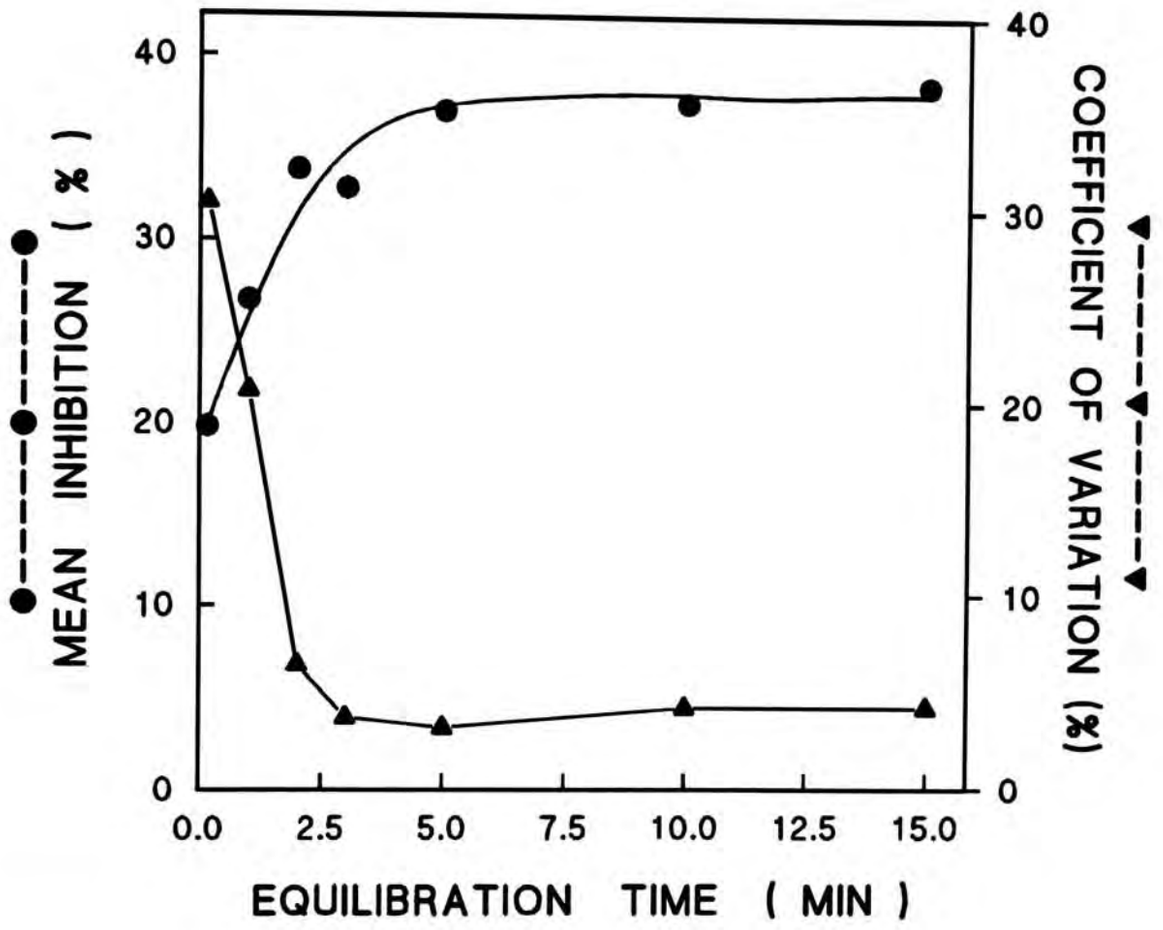


Figure 2-2: **Effect of equilibration time between plasma and C18 resin on recovery and precision performance.**

Effect of Na,K-ATPase activity on the sensitivity of ESTI measurement

In an attempt to improve the sensitivity of the present method, the effect of varying the Na,K-ATPase activity in the reagent mixture was studied using ouabain as the ESTI. A series of 28 aqueous ouabain standard solutions was prepared by appropriate dilution of a 1 mmol/l stock ouabain solution in volumetric flasks. The standard concentrations ranged from 20 nM to 0.1 mmol/l. The incubation of ouabain standard solutions with the Na,K-ATPase was performed on the Cobas Bio automatically for 30 sec before the measurement of the residual Na,K-ATPase activity as previously described. The reagent mixture was prepared to contain 150 U/l of Na,K-ATPase activity. This experiment was conducted with each ouabain standard in duplicate. The mean %I was plotted against each standard concentration. The ouabain concentration required to cause 50% inhibition of the enzyme activity (IC_{50}) was estimated from the standard curve. The above experiment was then repeated with Na,K-ATPase activity of 100, 50, 25 and 12.5 U/l.

IC_{50} was used to indicate the sensitivity of the ESTI measurement. There was no significant difference over the range of Na,K-ATPase activities studied in this experiment. IC_{50} values ranged from 2.6 to 5.2 μM. This implied that this experimental design can only be used to measure ESTI in the range of μM ouabain equivalent concentration, which is not sensitive enough compared to many established methods.

Effect of incubation time with Na,K-ATPase on the sensitivity of ESTI measurement

To improve the sensitivity of the present method, the effect of incubating the extract for different time periods with Na,K-ATPase was studied using ouabain and plasma extract. A series of 20 aqueous ouabain standard solutions was prepared by appropriate dilution of a 1 mmol/l stock ouabain solution in volumetric flasks. The standard concentrations ranged from 1 nM to 7 μM. The incubation of ouabain standard solutions with the Na,K-ATPase was performed as previously described except the procedure was automated on the Cobas Bio. The incubation mixture was

inside the cuvette rotor for the programmed time of 6 min. At the end of the incubation time, the Cobas Bio was programmed to continue with the measurement of residual Na,K-ATPase activity immediately without any delay. The %I was calculated and plotted against the ouabain concentration for each standard on a semi-logarithmic graph. The IC_{50} value was read from the curve. The above procedure was then repeated for incubation times of 0.25, 0.5, 1, 2, 3 and 4 h.

The effect of incubation time is shown on Figure 2-3. The %I of Na,K-ATPase activity increased with ouabain concentration in a dose dependent manner as expected. The IC_{50} value decreased with increasing incubation time for the first hour and then gradually plateaued after 2 h. There was no significant improvement when incubated for 3 and 4 h. The introduction of a 2 h incubation time before the measurement of residual Na, K-ATPase activity increased the sensitivity of the method 7 fold. The present method was able to measure ESTI in the nM range of ouabain equivalents that is comparable with established methods.

For the study of plasma extracts, a pooled plasma sample collected from healthy volunteers was processed as previously described. To increase the inhibitory activity of the plasma extract, the extract was concentrated 2 fold by reconstituting the residues with half of the required Buffer A. The extraction and concentration of plasma sample was performed in duplicates. The incubation of the concentrated plasma extracts with Na,K-ATPase was performed as described above. Incubation time periods tested were: 5, 15, 30 and 45 min; 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5 and 4.0 h. The %I of the Na,K-ATPase activity was calculated for each incubation time. The results were plotted against the incubation times on a graph as shown on Figure 2-4.

The effect of incubation time on the inhibition of Na,K-ATPase activity in a plasma ESTI extract was similar to that for ouabain standards. The introduction of a 2-h incubation time also improved the sensitivity of the method, but only 1.7 fold in magnitude. Prolonged incubation did not significantly improve it further.

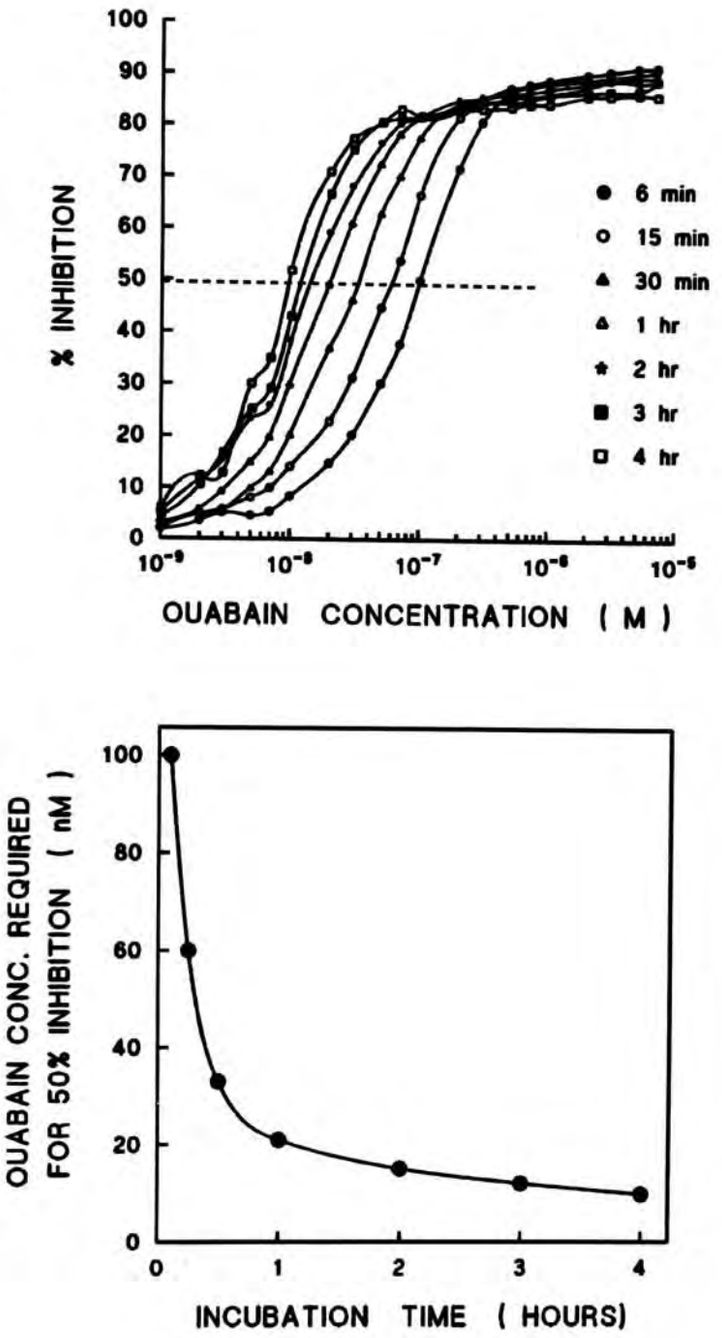


Figure 2-3: **The effect of incubating aqueous ouabain standards (top) with Na,K-ATPase for different time periods and the IC_{50} values at different incubation time duration (bottom).**

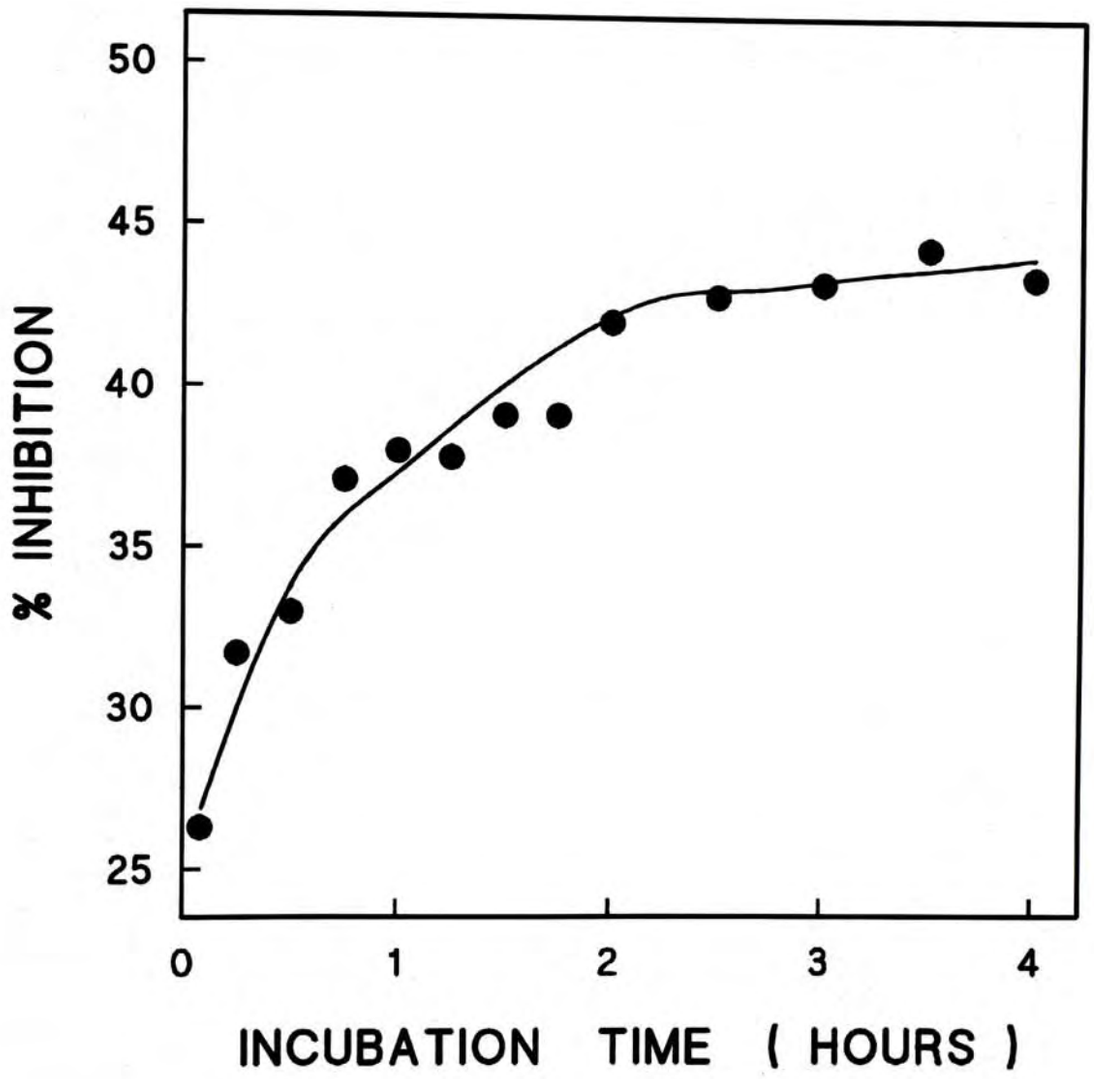


Figure 2-4: **The effect of incubation time on the % inhibition of plasma extract and purified Na,K-ATPase.**

Dose response characteristic of plasma ESTI

Plasma samples collected from two male healthy volunteers on the last day of a 7-day salt loading experiment were used. The extraction of ESTI, incubation with Na,K-ATPase and measurement of residual enzyme activity were performed as described in the METHODS section. A sample volume of 100, 150, 200 or 250 µl was loaded onto the Sep Pak cartridge in duplicate and extracted. The %I on the Na,K-ATPase activity was calculated and the mean result of the duplicates was plotted against the sample volume.

The dose response curves for the 2 plasma samples are shown on Figure 2-5. The %I of the Na,K-ATPase activity was linear and directly proportion to the amount of plasma sample loaded onto the Sep Pak cartridge. The linear regression equations for both dose curves were very similar indicating that the characteristics of the ESTI extracted from the 2 samples were also similar.

Analytical performance of the optimized method

Between batch precision performance

Five ouabain standard solutions with concentrations of 5, 10, 20, 30 and 50 nM were incorporated into each batch of measurements. Over a period of 9 months, there were a total of 84 batches. The precision performance of these standards was calculated and is shown in Table 2-6. At a concentration of 5 nM, the between-batch precision was 15%.

Sixty lithium heparin plasma samples were collected for a salt-loading experiment. Every sample was measured for plasma ESTI in duplicate as described in the METHODS section over a period of 2 weeks and the analytical coefficient of variation (CV) calculated for the 60 duplicates. The mean ESTI value was 11.9 nmol OE/l, and the CV was 11.8%. The precision performance was similar to the between-batch precision of the aqueous ouabain standards of similar concentration.

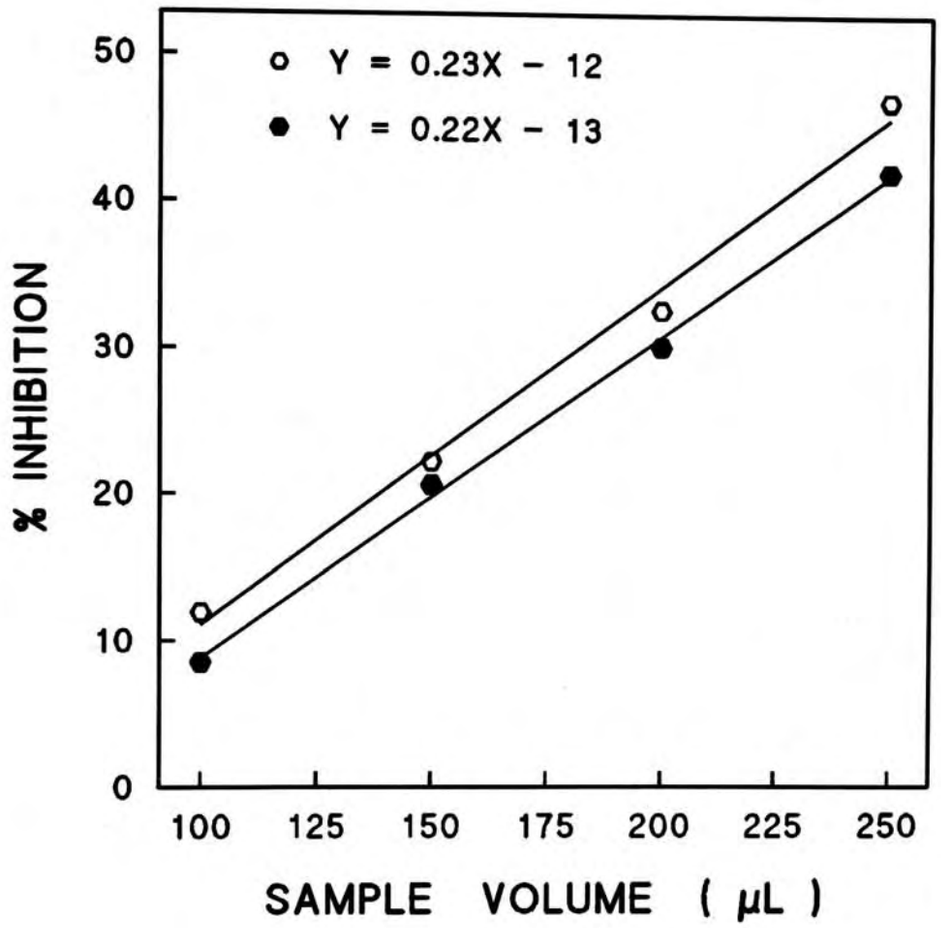


Figure 2-5: Dose response curves for 2 plasma samples.

Recovery studies

Recovery studies were performed using both aqueous ouabain standard solutions and lithium heparin plasma spiked with ouabain standards. The ouabain standard solutions described above were used. Each ouabain standard solution was extracted 10 times using 2 separate Sep Pak cartridges and analysed as described in the METHODS section. The results are shown in Table 2-7. The recovery ranged from 83% for the 5 nM standard to 110% for the 10 nM standard. The average recovery for the 5 standards was 98.6%.

To a pool of lithium heparin plasma collected from healthy adult volunteers, aqueous ouabain solution was added to produce plasma samples containing 0, 5,10, 20,30 and 50 nM of ouabain. Each plasma sample was extracted 5 times as described above. The results are also tabulated on Table 2-7. The recovery ranged from 132% for the 5 nM sample to 98% for the 50 nM sample. The positive bias was observed significantly for the samples with low ouabain, probably due to non-specific ESTI present in the sample pool. The average recovery was 117 %.

Table 2-6: Between batch precision performance of aqueous ouabain standards over a period of 9 months (n=84).

Ouabain concentration (nM)	Mean % Inhibition	Coefficient of Vairation (%)
5	14.3	15.3
10	27.0	13.8
20	49.3	4.5
30	63.6	3.2
50	75.4	2.3

Table 2-7: Recovery study for the established method using aqueous ouabain standard solutions and plasma samples spiked with ouabain

Ouabain conc. (nM)	% Recovery	
	Aqueous standards (n = 10)	Spiked plasma (n = 5)
5	83	132
10	110	128
20	101	118
30	99	109
50	100	98

Improving the sensitivity of the RIANEN method for the measurement of DLI

Attempts were made to reduce the lowest detection limit of the RIA assay so that the method could be used to measure lower concentration of DLI. The method of measurement was similar to that described in the METHODS section with several modifications. The sample volume was increased to 100 μl. Both the tracer and antibody volumes were varied between 50 to 200 μl. The incubation time was also varied between 30 and 60 min. A set of standard solutions with low concentration of digoxin was prepared by appropriate dilution of the 8.0 μg/l kit calibrator with the zero calibrator to give the following concentrations: 0.025, 0.050, 0.10, 0.20, 0.30, 0.50, 1.0, and 2.0 μg/l. By running 10 replicates of the zero standard, the lowest detection limit was determined as the mean + 2 standard deviation of the measured values. Pooled samples of healthy adult plasma and cord plasma were extracted by the Sep Pak cartridges as described in the METHOD section. One digoxin quality control sample with an assayed value of 1.0 μg/l was included in each set of modified conditions.

The results are tabulated in Table 2-8. The different protocols were able to lower the detection limits from 0.10 μg/l to as low as 0.03 μg/l. The prolonged incubation time and increased sample volume were most effective. The mean value of all the digoxin quality control samples was 0.96 ± 0.032 μg/l (n=9), ranging from 0.93 to 1.00 for all the protocols. However, the modified protocols detected lower DLI concentrations compared with the manufacturer's protocol. These results suggested that when the assay was made more sensitive for digoxin, conditions were less favourable for the cross-reacting ESTI. To optimise the measurable DLI, it was decided that the manufacturer's recommended protocol would be used for the rest of the study.

Table 2-8: Effect of modification of assay protocol on the detection limit for DLI in adult and cord-blood plasma pools

Volume used (µl)				DLI measured (µg/l)	
Sample	Tracer	Antibody	Detection Limit(µg/l)	Adult	Cord
Incubation time = 30 min					
40 **	200 **	200 **	0.10	0.61	0.86
100	200	200	0.03	0.32	0.41
100	100	200	0.05	0.24	0.28
100	50	200	0.06	0.16	0.23
100	200	100	0.09	0.24	0.30
100	200	50	0.08	0.29	0.33
Incubation time = 60 min					
100	200	200	0.03	0.19	0.30
100	100	200	0.03	0.17	0.19
100	50	200	0.05	<0.05	0.10

**Manufacturer's recommended protocol with proportionately reduced volumes.

Analytical performance of the modified CEDIA method

Precision performance

The within-batch precision study was performed by running 8 replicates of the 3 calibrators provided with the kit using the method described in the METHODS section. Between-batch precision was assessed by running 3 levels of digoxin quality control samples over a period of one month. To evaluate the difference in performance between the manufacturer's recommended protocol with the reduced reagent volume protocol, the between-batch precision was similarly studied using the recommended protocol for 2 weeks. Student's paired-t test was used to evaluate the difference among the 2 sets of data at the 95% confidence limit.

The precision performance of the method was summarised on Table 2-9. The CV for between-batch precision ranged from 4.3 to 8.8% and was similar to the manufacturer's specification. There was no significant difference between the results obtained from the recommended protocol using full reagent volume and the modified protocol using reduced reagent volume.

Accuracy performance

The lowest detection limit of the method was evaluated by analysis of 10 replicates of the zero calibrator and was defined as mean + 2 standard deviation of the measured values. A correlation study for the measurement of DLI was performed using 25 cord heparinized plasma samples collected randomly, irrespective of sex, from newborns whose mothers were not on digoxin. DLI was measured as described in the METHODS section.

The lowest detection limit was found to be 0.08 μg/l, which was better than the manufacturer's claim of 0.2 μg/l. DLI was detected in 23 out of the 25 cord plasma samples and the concentration ranged from 0.08 to 0.76 μg/l.

Table 2-9: Precision performance of the CEDIA digoxin method: within-batch study used 3 calibrators (A,B,C); and the 2 between-batch studies used quality control samples (D,E,F)

	Samples	Mean	CV %
Within-batch (n=8)	A	1.07	5.7
	B	2.03	2.4
	C	3.99	1.0
Between-batch full reagent (n=12)	D	1.26	7.3
	E	1.86	6.1
	F	3.70	3.7
reduced reagent (n=8)	D	1.31	8.8
	E	1.94	5.9
	F	3.74	4.3

The use of acidified urine samples for the measurement of urine ESTI

The aim of the project was to study the roles of urinary dopamine (DA) and ESTI in natriuresis. Urinary DA is stable in acidified urine samples. It would be more convenient if both parameters could be measured on the same urine sample. Thus, the effect of acid on the measurement of urine ESTI was studied.

Fresh urine samples collected from two healthy male adult volunteers were used. The sample was separated into three 4-ml aliquots. The pH of two aliquots was adjusted to between 1.0 and 2.0 using 1 M HCl solution. One acidified urine and the untreated urine were lyophilised 2 h after adjusting the pH. The second acidified aliquot was lyophilised 24 h after adjusting the pH. The lyophilised urines were reconstituted with 1 ml of deionized distilled water. Urines were extracted and analysed as described in the METHODS section from one subject.

The results are shown on Table 2-10. Acidification of the 2 urine samples increased the ability to inhibit Na,K-ATPase activity by over 200%. There was no significant difference between the acid treatment for 2 h and 24 h. However, the DLI was relatively unaffected.

The effect of DA in the measurement of urine ESTI

The effect of DA on Na,K-ATPase was studied systematically. A series of aqueous standards of DA were prepared by appropriate dilution of a 10 mmol/l stock DA solution in 0.1 M HCl using Buffer A. The concentration of the 18 working DA standard solutions ranged from 25 nM to 5 mmol/l. DA solutions were inclubated and the Na,K-ATPase Na,K-ATPase activity performed as described in the METHODS section.

To examine the concentration of DA in urine extracts, a urine sample was extracted 20 times after measuring the DA concentration. The methanol fractions were pooled and evaporated to dryness. The residues were reconstituted with 0.1 M HCl and the DA concentration in the Sep Pak eluants was measured.

Table 2-10: Effect of acid preservative on the measurement of urine ESTI.

Treatment	% Inhibition		% Increase		DLI (µg/l)
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1
Untreated	14.8	31.2			2.11
Acid 2 h	39.0	67.7	264	217	2.08
Acid 24 h	40.5	62.8	274	201	2.22

The comparison of the characteristic inhibition patterns of ouabain and DA is shown on Figure 2-6. The concentration of DA required to demonstrate inhibitory activity was 100 μM. The IC_{50} value for DA was about 300 μM. DA concentration in the Sep Pak eluants was only 1.6% of the original DA concentration. Thus it was concluded the interference of DA in the measurement of urine ESTI was negligible.

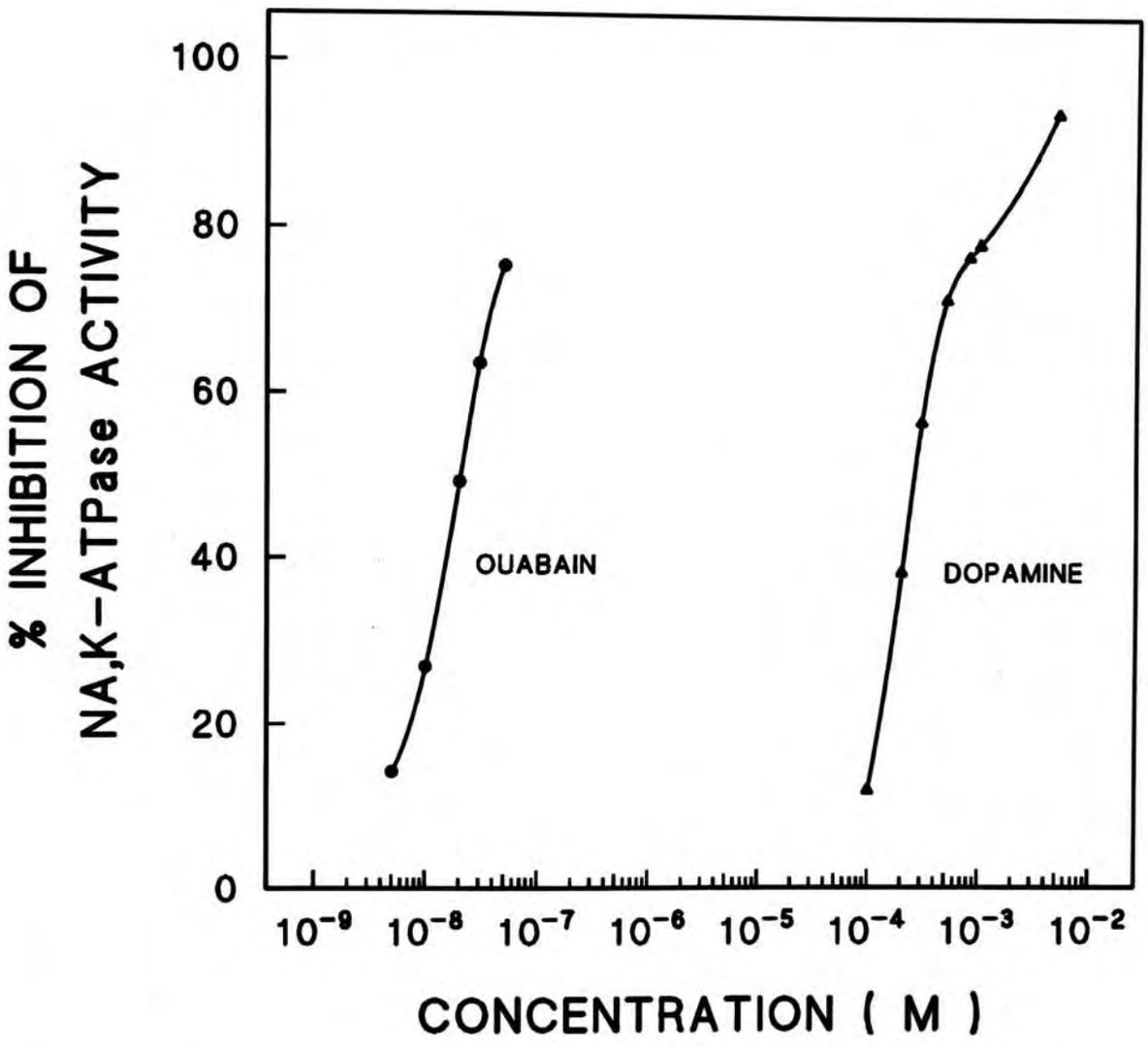


Figure 2-6: The inhibition characteristics of dopamine and ouabain.

DISCUSSION

Two methods were developed for the measurement of ESTI in this project. Since the nature of ESTI has not been established, it is recommended to use a combination of at least two methods for its measurement (Goto *et al* 1992). It is desirable for one of the methods to be based on inhibition of cellular sodium transport. In this study, the first method developed was based on the inhibition of purified dog kidney Na,K-ATPase and the second method on DLI using 2 different commercial available digoxin kits. To reduce the amount of other interfering substances, a preliminary purification procedure was developed using solid phase extraction.

Inhibition of purified Na,K-ATPase measured by enzyme-coupled reactions

In this method, Na,K-ATPase activity is measured spectrophotometrically by coupling to two other enzymes, PK and LD. This coupled enzyme reaction maintains an optimal ATP concentration for Na,K-ATPase activity as the ATP is regenerated by the subsequent reaction. As similar method has been used to demonstrate increased ESTI in subjects with essential hypertension (Hamlyn *et al* 1982). The present method has improved features compared to others reported in the literature. After the initial binding and inhibition of the ESTI on the enzyme, measurement of the residual Na,K-ATPase activities has been automated on a centrifugal analyser. The same method can also be easily automated on other discrete routine clinical chemistry systems. Unlike some methods based on inhibition of purified Na,K-ATPase, this method does not use radioactively labelled substrate or sophisticated instrumentation such as a luminometer. It is efficient, and 28 samples can be measured in 15 minutes. No concentration of the plasma sample is required and only 200 µl of plasma sample is necessary for duplicate analyses. This method can handle a large number of samples in a short period of time.

Different sources of Na,K-ATPase have been used, for example dog kidney (Hamlyn *et al* 1982), hog cerebral cortex (Gonick *et al* 1987) or pig kidney (Moreth *et al* 1987). Purified dog kidney Na,K-ATPase was chosen for this study because the sodium pump on the renal tubule is the target site of action for ESTI. This enzyme is

conveniently available commercially. It has been reported that the action of ESTI may be specific (Hamlyn *et al* 1988). Dog kidney purified Na,K-ATPase may not be a good substitute for human enzyme. It would be ideal to obtain human purified kidney enzymes, but it is not practical to obtain fresh sample for purification.

Methods based on this principle have been criticized. Na,K-ATPase is a complex and membrane-bound protein. It requires many ligands and substrates for cycling to occur, and for the binding of cardiac glycosides (Woolfson *et al* 1994). Purified enzyme is not embedded in the membrane and it becomes susceptible to inhibition of substances that do not have an effect *in vivo*. The situation can be exaggerated when these inhibitors are concentrated many fold after purification processes. The use of an optimal ATP concentration to measure the effect of Na,K-ATPase activity also does not reflect the physiological condition. The method described here does not require concentration of the sample, and the solid phase extraction purification procedure reduces interfering substances. Furthermore, the view that ESTI must be a cardiac glycoside has not been established. Therefore, the present method can be used to demonstrate the presence of ESTI in biological samples and to show changes of inhibitory activities after experimental maneuvers. Practically, very few methods reported in the literature can ensure that the measured ESTI reflects *in vivo* activity.

In establishing the reaction conditions for measuring Na,K-ATPase activity, concentrations of sodium, potassium, magnesium, ATP, and EGTA were optimized (Figure 2-1). The optimal concentrations established were similar to those reported in the literature using a similar source of enzyme.

ESTI are reported to be protein-bound (Valdes & Graves 1985). Different methods of pretreatment were used to disrupt the protein matrix of the samples so that the bound ESTI could be released to be measured more efficiently. These methods included a combination of dilution of the sample with water, acidification to deproteinize the sample, boiling, and extraction with organic solvents (Hamlyn *et al* 1982, Lau & Valdes 1988, Giunta *et al* 1990). In this study, these methods of pretreatment were examined systematically. However, the results showed that these

pretreatment methods before the Sep Pak extraction could not improve the recovery of ESTI from both cord plasma and normal adult heparinized plasma (Tables 2-3, 2-4a and 2-4b). On the contrary, some of the methods lowered the recovery dramatically. For example, boiling of the sample appeared to have denatured ESTI in the sample. Boiling of a larger volume within a short period of time preserved ESTI, probably due to uneven heating of the sample. With prolonged heating, inhibitory activities of the samples were not observed. Denaturing of ESTI by boiling had been reported (Balzan *et al* 1984, Moreth *et al* 1987). On the other hand, ESTI had been reported to be heat-stable (Devynck *et al* 1983). The present results indicated that solid phase extraction is sufficient to overcome this protein-bound interaction. Therefore, none of these pretreatment methods was considered further in the method development.

Attempts were also made to remove lipids adsorbed on the C18 resins before the methanol elution. Lipids were reported as non-ouabain-like inhibitors (Goto *et al* 1992). Thus, removal of lipids could improve the specificity of the eluted ESTI. However, these attempts resulted in poor precision of the amount of ESTI eluted. This phenomenon could probably be due to the interaction between the organic solvents and the C18 resins. This interaction could damage structural integrity of the resin affecting the reproducibility of ESTI adsorption onto the resins. Furthermore, selective removal of lipids from the samples assumes that ESTI must be a cardiac glycoside and again this had not been absolutely established.

In the course of evaluating the solid phase extraction of plasma samples, it was observed that precision performance was unsatisfactory. Automation of measuring Na,K-ATPase activity on Cobas Bio has good precision. Between-batch precision CV for measuring a 100 U/l enzyme solution (n=20) was better than 5%. However, within-batch precision CV for measuring ESTI extracted from a plasma pool was higher than 20% (n=10). Preliminary investigation indicated that the Sep Pak extraction step caused the precision problem. By allowing an equilibration time of 5 minutes between the plasma sample and the C18 resins, both the recovery and precision performance improved dramatically (Figure 2-2). Prior to this finding, the

sample was introduced to the Sep Pak cartridge under vacuum. The rate at which the sample passed through through the Sep Pak cartridge was difficult to control. This rate depended on many factors, for example, condition of the cartridge, viscosity of the sample, strength of the vacuum etc. Thus, the amount of ESTI adsorbed onto the resins could vary depending on these conditions resulting in poor precision. With the new procedure, a measured volume of sample was introduced slowly with a syringe. The sample was allowed to stay inside the resin compartment of the cartridge for 5 minutes before deionized distilled water was used to wash away the proteins and electrolytes. During this equilibration period, it is possible that interaction between the resins and the protein-bound ESTI reaches an equilibrium state. As a result, the amount of ESTI adsorbed onto the resin is proportional to the concentration of ESTI present in the sample. Thus, both the recovery and precision performance improved. This phenomenon had not been reported in the literature. Although the use of Sep Pak cartridges to extract ESTI has been popular, the analytical performance was rarely reported.

Many methods developed to measure ESTI were able to detect ouabain in nanomolar concentration (Moreth *et al* 1987, Kelly *et al* 1988). These methods require the use of radioactive isotopes or sensitive techniques like bioluminescence. Initially, the enzyme-coupled method, as developed in this project, was only able to measure ouabain at micromolar level. Decreasing the amount of Na,K-ATPase in the reagent system did not have a significant effect. It was noticed that sensitive methods usually incorporated an incubation step in which ESTI and the Na,K-ATPase were incubated in a potassium-free medium for several hours (Kelly *et al* 1985). Thus, a systematic study of the effect of such an incubation step on the sensitivity of the present enzyme-coupled method was done. As a result of the introduction of a 2-hour incubation step the sensitivity improved 7 fold (Figure 2-3). With this incubation step, the method is comparable with radioreceptor assays in terms of sensitivity and was able to measure ouabain in nanomolar concentration. However, the effect on measuring plasma ESTI was less dramatic. There was an almost 2 fold increase in sensitivity (Figure 2-4). Such a discrepency in response compared with ouabain standards indicated that the plasma ESTI may not be an ouabain-like entity or the extract contained a mixture of

different inhibitors, possibly including ouabain. Ouabain has been reported to be present in normal human samples (Ludens *et al* 1991).

Analytical performance of the method was satisfactory. The between-batch CV over a period of 9 months ranged from 2% to 15% for ouabain standard solutions of 5 to 50 nM (Table 2-6). The CV was higher with lower concentrations of ouabain. For plasma samples, the analytical CV for duplicate analyses was found to be less than 12% for a mean ESTI concentration of 11 nM ouabain equivalent. When ouabain standards were spiked into a plasma pool, a recovery study showed that there was a high bias of 132% for low ouabain concentration (Table 2-7). The recovery was close to 100% when the spiked ouabain standards were more than 30 nM. The high bias with low ouabain standard may be due to the presence of non-ouabain-like ESTI present in the plasma samples. A dose response study showed that the inhibition of Na,K-ATPase activity was linear and proportional to the amount of plasma sample loaded on the Sep Pak cartridge.

DLI

The use of DLI as one of the methods to measure ESTI is popular. Most commercially available digoxin kit use antibodies that are directed against a specific portion of the steroid moiety of the digoxin molecule (Soldin 1986). Subjects who had not been on digoxin therapy were reported to have measurable DLI, for instance: neonates, pregnant women, patients suffering from hypertension, renal failure, congestive heart failure etc. It was suggested that DLI could be different in various conditions.

There are conflicting reports on the validity of using this approach to study ESTI. A significant relationship between the biological activities of ESTI and DLI has been reported by some research groups; while others found dissociation between the biological activity and DLI (Woolfson *et al* 1994). It has been claimed that non-specific binding of digoxin antibodies to endogenous compounds which do not possess cardiac glycoside-like characteristics has probably contributed more to the confusion in this field than any other single factor (Wechter & Benskas 1990). In this

study, the measurement of DLI in samples was preceded by the extraction step using Sep Pak cartridges. Since the Sep Pak extraction was optimized for maximum inhibitory activity, the presence of cross-reacting but non-inhibiting species was reduced. The Sep Pak eluants were not concentrated, thus supra-physiological concentrations of analytes were not expected. The present method improved the measurement of ESTI by DLI.

Two different commercial digoxin kits were used in this study. The unexpected discontinuation of the RIA kits forced the change to the other HEIA method. However,the change has been beneficial, as the new method can be automated on routine clinical chemistry analyzers. Precision performance was better compared with the manual RIA method. A correlation study between the two reagent kits was not possible as RIA kits were not available. The concentration of DLI by the Cedia HEIA in cord plasma was similar to that of the RIA method.

An attempt was made to improve on the lowest detection limit of the RIA method. This was achieved by varying the ratio between sample, tracer and antibody volumes, as well as the incubation time. The detection limit for digoxin was lowered from 0.10 µg/l to 0.03 µg/l (Table 2-8). However, the concentration of DLI in adult and cord plasma samples detected was also reduced by these modifications. Sensitivity of the digoxin assay was improved by providing a more favorable reaction condition to digoxin. Such a condition, logically, reduces the opportunity of other cross-reacting species to bind onto the digoxin antibody. Thus, the manufacturer's recommended protocol was maintained in order to facilitate the measurement of DLI and no attempt to improve the detection limit for the HEIA kit was made. Instead, efforts were made to reduce the manufacturer's recommended reagent volume for economical reasons and it was possible to do this without affecting analytical performance (Table 2-9).

Urine ESTI

Measurement of urine ESTI can be used to assess the production of ESTI over a period of time. This has an advantage over measurement of plasma ESTI, which only

reflects the ESTI concentration at one point in time. The 2 methods developed in this study can be adapted to measure urine ESTI.

The objective of this thesis is to study the roles of both DA and ESTI in natriuresis. It would be more convenient if both urine DA and ESTI could be measured in the same urine collection. Since acid preservative has to be used for collection of urine DA, it is necessary to study the effect of acid preservative on urine ESTI. It was interesting to observe that acid preservative increased the inhibitory activity of urine without DLI (Table 2-10). These results suggest that acid activated some inhibitors which have different immunoreactivity with the digoxin antibodies. There have been reports showing the presence of ESTI of different molecular weights in urine (Gonick *et al* 1987). It is possible that the large molecular weight ESTI are hydrolyzed into smaller molecules in an acidic solution, increasing the inhibitory activities.

Urine DA has also been reported to have an inhibitory effect on Na,K-ATPase activity. It is important to study whether the measured urine inhibitory activity is due to DA. The concentration required to inhibit Na,K-ATPase activity was supra-physiological and was not seen in any of the studies conducted in this project (Figure 2-6). Furthermore Sep Pak extraction removed more than 98% of DA from urine. Therefore, it can be safely concluded that the ESTI measured in urine extracts is not DA.

Concluding remarks

Two methods were developed for the measurement of ESTI for this project. The method based on the inhibition of purified dog kidney Na,K-ATPase is sensitive with satisfactory analytical performance. It can measure ouabain in nM concentration without using radioactive isotopes or complicated instrumentation. The method based on the measurement of DLI is convenient and has been automated for improved precision performance. The use of solid extraction to purify ESTI before measurement reduces the interferences by other non-specific species.

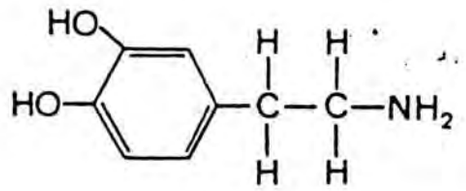
CHAPTER 3

MEASUREMENT OF URINARY FREE DOPAMINE

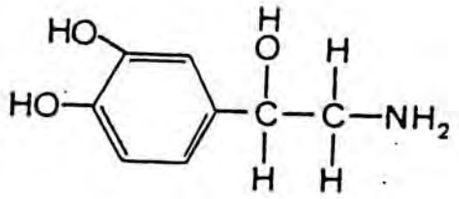
I. LITERATURE REVIEW

PROPERTIES OF DOPAMINE FOR MEASUREMENT METHODS

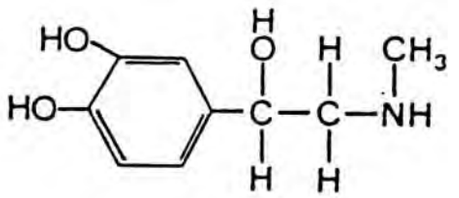
The measurement of dopamine (DA) has been based on the biological, physical and chemical properties of DA (Nyyssönen & Parviainen 1989). It shares a common molecular formula with noradrenaline (NA) and adrenaline (A) as shown on Figure 3-1. Hormonal actions of catecholamines (CATS) provided the basis for the early bioassays. The presence of both amino and phenolic groups provides the CATS with the amphoteric properties and high solubility in water. Such an ionic nature enabled the extraction, purification and separation of CATS by ion-exchange resin. The characteristic catechol ring structure (1,2-dihydroxybenzene) makes the CATS sensitive to oxidative agents which convert it to quinone forms. This property is commonly used for the detection of DA. However, this unique feature also requires the addition of reducing substances as preservatives to protect biological samples before and during the analytical processes. The ring structure also gives native fluorescence. The primary amino group of DA reacts with other chemicals to produce fluorescent derivatives. Fluorescence has been used for sensitive quantitative measurement. Furthermore, the 3,4-dihydroxyphenyl structure of CATS allows formation of complexes with other compounds, for example alumina and boric acid and this provides specific extraction procedure even at nanomolar concentration in different biological samples. Methods developed to measure plasma samples can be applied for the measurement of CATS in urine samples. However, the reverse is not true as the urinary CATS concentration are 10 - 100 fold higher than the plasma levels.



Dopamine



Noradrenaline



Adrenaline

Figure 3-1: Molecular structures of catecholamines

PRESERVATIVES USED IN THE URINE COLLECTION FOR MEASURING FREE DA

DA is unstable under neutral and alkaline conditions like other CATS. CATS are easily oxidized by the presence of free radicals. The formation of free radicals can be stimulated by ultraviolet radiation, elevated temperature and the presence of transition-metal cations. Thus, during the process of urine collection, the samples are kept in the cold, in the dark, in the presence of chelators and reducing substances (Hugh *et al* 1987). CATS are stable at pH values between 2 and 5.

It has been a common practice to use acid preservatives for urine samples. A large variety of acids have been used, for example difference concentrations of hydrochloric acid (HCl), sulphuric acid (H_2SO_4) and acetic acid. Examples of common acid preservatives used are listed on Table 3-1. Urine samples have also been collected into plain bottles (de Jong *et al* 1987), bottles containing glutathione / EGTA (Demassieux *et al* 1981) or sodium metabisulphite (Anton & Sayre 1962, Westerink & ten Kate 1986). Research groups using lower concentrations of acids with/without other preservatives tended to adjust the final pH to approximately 2 to 4 by stronger acids like concentrated HCl (Fotl *et al* 1987), 85% phosphoric acid (de Jong *et al* 1987) or concentrated formic acid (Westerink & ten Kate 1986) before storage. Anderson *et al* collected 24 h urine in 6 M HCl and before storing the samples at low temperature, solutions of sodium metabisulphite, EDTA and an internal standard were added to a fixed volume of urine (Anderson *et al* 1988). This not only preserved the sample, but also allowed the changes during storage to be evaluated by measuring the amount of internal standard remaining in the sample.

There is no consensus in the literature on the appropriate type or optimal concentration of acid that should be used to preserve urine samples except that the pH should be low. The choice of acid and its concentration has rarely been explained in the literature. Thirty years ago, the use of 0.4 M perchloric acid was observed to increase urinary CATS significantly at room temperature and 5 °C (Anton & Sayre 1962). However, this phenomenon has not been studied systematically. High concentrations of inorganic acids might hydrolyse conjugates of CATS giving falsely

high free CATS concentrations (Euler & Von Lishajko 1961, Henry & Pratt 1990). Most of the urinary DA is in conjugated forms, 5 times higher than that of the free DA (Westerink & ten Kate 1986). The optimal conditions to preserve and store free DA in urine should be established in order to avoid possible artifacts due to hydrolysis of conjugated DA. Lastly, a report questioning the need for any special collection conditions for urinary CATS has added to the confusion (Konstantinides *et al* 1988).

Table 3-1: Examples of acid preservatives used of collection of urine for free catecholamine measurement

Preservatives:	References:
Concentrated HCl	Davidson & Fitzpatrick 1985, Wu & Gornet 1985
6 M HCl	Parker *et al* 1986, Fotl *et al* 1987, Peaston *et al* 1988, Lee *et al* 1989
5 M HCl	Nair & Munk 1987, Critchley *et al* 1989
3 M HCl	Oates *et al* 1980, Güllner H G *et al* 1982
2 M HCl	Lundberg *et al* 1988
25 mM HCl	Smedes *et al* 1982
1 M H_2SO_4	Moerman & de Schaepdryver 1984
0.5 M H_2SO_4	Weinkove 1991
33% glacial acetic acid	Moyer *et al* 1979

SAMPLE PRETREATMENT PROCEDURES BEFORE THE MEASUREMENT OF URINARY CATS

Since the concentration of CATS in urine is low and sensitive methods are required to perform quantitative measurement, sample pretreatment is required to remove interferences from the final reaction matrices. Organic solvent has been used to extract urinary CATS which are then back-extracted into acid aqueous solution. Most of the methods are based on complex formation between the hydroxyl groups of CATS with different resins at alkaline pH. The amphoteric properties of CATS also allow the extraction using ion-exchange resins.

Alumina has been popular for a long time (Aston & Sayre 1962, Davidson & Fitzpatrick 1985, Fotl *et al* 1987, Anderson *et al* 1987, Weinkove 1991). Urinary CATS were adsorbed onto the alumina in an alkaline buffer at an optimal pH of 8.6. However, the alkaline pH enhances oxidation of CATS leading to low recovery. Thioglycolic acid, EDTA, EGTA, sodium metabisulphite, ascorbic acid or a combination of the above have been used to protect the CATS during the process (Anton & Sayre 1962, Hugh *et al* 1987, Davidson & Fitzpatrick 1985). It was reported that the combination of 2.8 mM of ascorbic acid and sodium metabisulphite can protect the CATS for 5 h at the pH of 8.6 (Davidson & Fitzpatrick 1985). The buffer was then washed away with water or dilute buffer and CATS were eluted with an acid solution, for example acetic acid (Anderson *et al* 1988, Weinkove 1991), hydrochloric acid (Davidson & Fitzpatrick 1985) and perchloric acid (Aston & Sayre 1962). Ethyl acetate washing has also been used to remove electro-active species after the alumina extraction (Davidson & Fitzpatrick 1985) to improve the specificity. The recovery by the alumina extraction ranged from 76% to almost 100% (Aston & Sayre 1962, Davidson & Fitzpatrick 1985, Nyyssönen & Parviainen 1989). It was claimed that alumina purification alone was sufficient for the subsequent chromatographic measurement (Foti *et al* 1987).

Boric acid gels and boric acid affinity columns also form a stable complex with CATS at alkaline pH and have been used for the extraction of urinary CATS (Moyer *et al*

1979, Oka *et al* 1982, Peaston 1988). Commercial columns of immobilized boric acid or phenylboric acid are available and the linkage of boric acid to the support matrix may influence its adsorptive / selective properties for CATS (Rosano *et al* 1991). Cation exchange resins such as Bio-Rex 70 were often used (Weicker *et al* 1984, Pillai 1987). Borate could be used to selectively elute CATS from ion-exchanger rather than the less-specific elution by pH adjustment (Odink *et al* 1986). Furthermore, some of these resins were used with alumina to improve the specificity. A combination of phenylboronic acid followed by alumina was reported to be the best among 4 extraction methods studied (Wu & Gornet 1985). An efficient solvent extraction method using ion-pair formation with diphenylborate under alkaline condition has also been reported (Smedes *et al* 1982).

Automation of the extraction process was also attempted using more sophisticated instrumentation based on column chromatographic techniques. On-line pre-column extraction methods using either alumina or dihydroxyborylsilica column was successful (de Jong *et al* 1987). An alkaline low buffer concentration mobile phase was used to adsorb the CATS onto the pre-column. The system then switched to an acidic high buffer concentration mobile phase to elute the CATS. It finally switched to another column and a third mobile phase for the separation of individual CATS. Another automated commercial system was also reported using "automated sequential trace enrichment of dialysate" technique (Green *et al* 1989). The urine sample was not dialysed but enriched in a stainless steel cartridge using proprietary resin. The interference was washed away. The CATS was eluted and injected into another column for separation. These automated systems provided good recovery and precision performance, but were complicated and expensive.

METHODS OF MEASUREMENT

Bioassays for the measurement of CATS

Historically, CATS were discovered as a result of their pharmacological properties on biological systems. Bioactivity of CATS on tissues, isolated organs and even animals were used for quantitative measurement (Gaddum 1959). For example, the contraction of rat stomach strip preparation was sensitive to 1 to 10 ng of E (Armitage & Vane 1964); and the perfusion pressure of an isolated central artery of a rabbit ear increased in dose response with NE and E concentrations (de la Lande & Harvey 1965). Bioassays were sensitive but not specific (Vane 1966). An isolated guinea pig heart preparation was developed to measure less than 1 ng of NE and E (Booker *et al* 1962). But the same preparation was also reported to measure angiotensin (Watson & Booker 1965). The presence of contaminants that could have CATS-like activity, or potentiate the effects of CATS, or have antagonist properties make the bioassay methods unreliable. Thus, the extraction procedure was essential for accurate measurement.

To improve the specificity of bioassays, superfusion techniques were developed. Tissues with different sensitivities to individual CATS were joined in series. For example, rat stomach strip was superfused with a chick rectum (Armitage & Vane 1964). The chick rectum was about 100 times more sensitive to E than to NE. The degrees of contractions in the 2 tissues from test solutions were compared with that of E and NE standards. Proportions of E and NE in the test solutions were calculated by solving simultaneous equations. This technique combined sensitivity and specificity by careful selection of the tissue preparations. However, the problem of contamination was not resolved.

Although bioassays were claimed to be cheap and did not require sophisticated instrumentation, they have been replaced by chemical and biochemical methods for the measurement of CATS. Working with isolated tissues and organs demanded more technical skill and suffered from the large variations inherent with biological systems.

Furthermore, the problem of contamination and non-specificity could only be resolved by chemical and biochemical techniques.

Colorimetric method for the measurement of CATS

Colorimetric methods for the measurement of CATS are based on the formation of adrenochromes from the oxidation of CATS in the presence of oxidizing agents, for example iodine, alkaline substances and sunlight. There are also reaction products with ninhydrin and diazobenzenesulphonic acid. However, colorimetric methods are neither specific nor sensitive for the determination of CATS (Kågedal & Goldstein 1988).

Fluorometric methods for the measurement of CATS

The catechol ring structure makes CATS naturally fluorescent at an excitation maximum of 200 to 220 nm or 280 to 300 nm and an emission maximum of 310 to 330 nm (Nyyssönen & Parviainen 1989). Although the characteristic fluorescence of CATS had been used for a fluorometric assay (Lowry 1948), the presence of other natural fluorescent compounds with similar fluorescence spectra in biological fluids rendered this method to be non-specific. Therefore, chemical methods to obtain different fluorescent derivatives for sensitive measurement were developed.

Early assays were based on the oxidation of CATS under alkaline conditions to form fluorescent trihydroxyindole products called adrenolutins. Initially, only NE and E could be measured in urine samples (Jacobs *et al* 1961, Anton & Sayre 1962, Kahane & Vetergaard 1965). Examples of oxidizing agents were potassium ferricyanide (Anton & Sayre 1962, Moyer *et al* 1979) and manganese dioxide (Uuspää 1963). Later modification of the method to include iodine as the oxidizing agent allowed for the additional measurement of DA (Carlsson & Waldeck 1958, Oka *et al* 1982). Stabilizers were required in the assay as the fluorescent products were unstable. Ascorbic acid was commonly used (Anton & Sayre 1962, Moyer *et al* 1979). However, the fluorescence blank was found unstable and other stabilizers were included to improve the situation, for example, ethylene diamine or propylene diamine plus ascorbic acid (Euler & Von Lishajko 1961) and dimercaptopropanol or cysteine hydrochloride (Häggendal 1963). Measurement of the individual CATS was achieved

by paper chromatography, by oxidation at different pH or by the differences in the activation and fluorescence spectra (Häggendal 1966). The detection limit of these methods was in the nanogram range.

Ethylenediamine condensation was another group of fluorometric methods based on the condensation reaction between oxidized CATS with organic primary amines to produce polycyclic fluorescent compounds (Natelson *et al* 1949, Weil-Malherbe & Bone 1952). The product was more stable than the trihydroxyindole assays. These methods were more sensitive and had detection limits in the picogram range (Kågedal & Goldstein 1988). However, they were not commonly used as compared to the trihydroxyindole methods probably because of the lack of specificity (Häggendal 1966).

The measurement of urinary CATS with fluorometric assays was gradually replaced with new chromatographic methods (Rosano *et al* 1991). On the other hand, the principle of these methods is still used with the chromatographic technique for detection of the separated individual CATS.

Radioimmunoassays for the measurement of CATS

Although radioimmunoassay for the measurement of CATS was reported (Raum & Swerdloff 1981), the application for urinary DA has not been found. This technique suffers from the non-immunogenic nature of CATS and they must be conjugated with a carrier protein for the production of antiserum. Since the catechol ring is susceptible to oxidation during the conjugation process, the usefulness of this method has been limited (Knoll & Wisser 1984).

Radioenzymatic methods for the measurement of CATS

Radioenzymatic methods have been developed for the sensitive detection of plasma CATS whose concentrations are lower than that of the urine samples. They require very small sample volume and no pretreatment. The principle has been used for the measurement of DA in urine samples (Casson *et al* 1983, Harvey *et al* 1984) and tissues (Da Prada & Zürcher 1976). The enzyme catechol-o-methyltransferase,

purified from liver homogenates, is used to transfer radioactive methyl groups from S-adenosyl-methionine to endogenous catecholamine acceptor molecules to form radioactive o-methyl catecholamines. The radioactive o-methyl derivatives are extracted from the reaction mixture and the products separated by thin-layer chromatography. Accuracy of the method can be improved by radioactively labeled internal standard (Johnson *et al* 1980). CATS have to be extracted from the urine samples before the measurement as the enzyme system is sensitive to inhibitors (Casson *et al* 1983, Harvey *et al* 1984). The detection limit of DA was claimed to be 32 fmoles (Da Prada & Zürcher 1976). Despite its sensitivity, the method is tedious, time-consuming, involving the use of radioactive isotopes and expensive (Foti *et al* 1987). Thus, this method is not extensively used for the measurement of urinary CATS.

Chromatographic methods for the measurement of CATS

Chromatographic methods are the most commonly used methods for the measurement of urinary CATS. Individual CATS can be separated by the partition of analytes between a stationary and a mobile phase. The specificity of the method is better than any of the above methods as the individual analytes can be identified by the specific retention times.

Gas liquid chromatography is based on the partition of the analytes between a gaseous mobile phase and a liquid stationary phase. This technique has been used for the sensitive and specific measurement of plasma CATS, especially when coupled with mass spectrometer for detection (Nyyssönen & Parviainen 1989). Recently, application of this technique for urine samples was also reported (Graham *et al* 1993). However, the use of gas liquid chromatography to measure urinary CATS has not been popular, probably because of the need for derivatization of the analytes to volatile compounds and the more complicated /expensive instrumentation required.

High performance liquid chromatography (HPLC) is a technique to separate analytes between a liquid mobile phase and a stationary phase. An HPLC system requires a liquid delivery pump to move the mobile phase through a column packed with the

stationary phase under high pressure for fast separation. The column eluant is connected to the detector for quantitation. Sample pretreatment is required for HPLC measurement of CATS as described in the previous section "**sample pretreatment procedures before the measurement of urinary CATS**". Dihydroxybenzylamine (DHBA) has been the most popular internal standard in this group of methods (Nyyssönen & Parviainen 1989).

For the measurement of urinary DA, the popular stationary phase material is octadecylsilane (C18) particles. Reversed phase C18 chromatographic systems with ion-pair reagent are commonly reported in the literature (Rosano *et al* 1991). Examples of some of these chromatographic systems are listed in Table 3-2. The mobile phase is usually more polar than the stationary phase. Different buffer systems, such as phosphate, acetate/citric acid or formate, are used to maintain the pH of the mobile phase from 2.3 to 4.8. The pH and ionic strength of the mobile phase determine the ionization of the solutes in the system, thus affecting the partition of the solutes between the stationary and mobile phases (Moyer & Jiang 1978, Kontur *et al* 1984). An increase in pH increases ionization of the solutes and reduces the retention time with the non-polar stationary phase; while a decrease in pH suppresses ionization and enhances the interaction with the stationary phase prolonging the retention time.

A common problem with reversed phase HPLC is that the fast eluting NA cannot be separated from the solvent front and uric acid. Ion-pair agents are included in the mobile phase to solve this problem. These ion-pair agents possess both hydrophilic and hydrophobic functional ends. The hydrophobic ends adsorb onto the non-polarstationary phase while the hydrophilic ends interact with the protonated CATS due to electrostatic interaction (Krsulovic *et al* 1981). Retention times are made longer with increasing concentration enabling the separation of NA from the solvent front. Common examples of ion-pair agents are octanesulfonate and heptanesulfonate and the effective concentration ranges from 0.35 to 4 mM. Organic modifiers, such as methanol and acetonitrile ranging from 2 to 30%, are added to fine-tune the retention time. These organic solvents attenuate the interaction between the solutes and the stationary phase resulting in shorter retention time with increasing concentration of

the modifiers (Moyer & Jiang 1978). Furthermore, the use of a different buffer system with ion-pair agents can affect the stability of the stationary phase. It was claimed that the use of formate buffer was preferable over phosphate, acetate or citrate buffers because the formate buffer produced long-term stability for the HPLC packing material (Peaston *et al* 1988).

EDTA is often added to the mobile phase to chelate electroactive metal ions present in the mobile phase and this reduces noise in the electrochemical detector (Nyyssönen & Parviainen 1989). Amines are also used to saturate "active sites" of the stationary phase so that variation of inter-column performance can be reduced. Triethylamine has been commonly used. However, development of new technology to end-cap surfaces of the packing material makes the use of amines in the mobile phase unnecessary.

The use of ion-exchange chromatography to separate urinary CATS is less popular. Compositions of the mobile phases are simply acidic buffers with added EDTA for those applications using electrochemical detector. A few examples are listed in Table 3-2. Early applications were hampered by deficiencies in column reproducibility resulting in loss of the separation properties (Rosano *et al* 1991). There has been interesting reports that semi-irreversible loading of C18 columns with detergents altered the reversed phase column to behave like an ion-exchange mechanism (Mefford 1987, Mefford *et al* 1987). Although this enhancement managed to impart a 3-fold relative increase in the analytical signal for E versus NA, there was no advantage for the measurement of DA.

HPLC systems coupled with fluorometric measurement are based on the methods described in the section "**Fluorometric methods for the measurement of CATS**". Few methods managed to make use of the native fluorescence (Anderson *et al* 1981). Majority of the methods were based on pre-column or post-column derivatization of the CATS. For example, post-column adaptations included ethylenediamine condensation and trihydroxyindole method; pre-column methods included 1,2-diphenylethyleneamine treatment, dansylation, and derivatization of NA and DA with

Table 3-2: Examples of stationary and mobile phases used in the HPLC method for the measurement of urinary CATS.

Stationary Phase	Mobile Phase	Organic modifiers	Additives	References
Reversed phase C18	Phosphate buffer pH 4.8, with heptanesulfonate	5% methanol	EDTA	Moyer *et al* 1979
Reversed phase C18	Phosphate buffer pH 2.3, with pentanesulfonate	2% acetonitrile	nil	Oka *et al* 1982
Reversed phase C18	Acetate/ citric acid buffer pH 3.0, with octanesulfonate	4% methanol	di-n-butylamine EDTA	Weicker *et al* 1984
Reversed phase C18	Phosphate/ citric acid buffer pH 4.1, with octanesulfonate	12% methanol	EDTA	Moerman & de Schaepdryver 1984
Ion-exchange	Phosphate buffer pH 3.5	nil	EDTA	Wu & Gornet 1985
Reversed phase C18	Phosphate buffer pH 3.5, with octanesulfonate	nil	EDTA	Davidson & Fitzpatrick 1985
Reversed phase C18	Monochloroacetic acid and citric acid pH 3.0	3.75% acetonitrite	Diethylamine EDTA	Parker *et al* 1986
Ion-exchange	Phosphate buffer pH 2.31	10% methanol	EDTA	de Jong *et al* 1987
C8 resin coated tri-n-butyl-phosphate	Phosphate/ perchloric acid pH 2.5	nil	EDTA	de Jong *et al* 1987
Reversed phase C18	Phosphate buffer pH 3.5, with laurylsulphate	25% methanol	EDTA	de Jong *et al* 1987
Reversed phase C18	Phosphate/ citric acid buffer pH 3.1, with heptanesulfonate	11% methanol 4% acetonitrile	nil	Huge *et al* 1987
Reversed phase C18	Citrate/ formic acid buffer pH 3.1, with octanesulfonate	nil	Diethylamine EDTA	Fosti *et al* 1987
Reversed phase C18	Formate buffer pH 4.0, with octanesulfonate	5% methanol	Triethylamine EDTA	Peaston *et al* 1988
Reversed phase C18	Phosphate buffer pH 3.5, with dodecylsulfate	30% acetonitrile	EDTA	Anderson *et al* 1988
Reversed phase C18	Phosphate buffer pH 4.0, with octanesulfonate	4% acetonitrile	EDTA	Crawford *et al* 1990
Reversed phase C18	Phosphate/ citric acid pH 3.2, with heptanesulfonate	9.5% methanol 3.5% acetonitrile	EDTA	Weinkove 1991

o-phthalaldehyde (Rosano *et al* 1991). These methods are sensitive and specific, however, they have not been used extensively.

Electrochemical detection is the most popular detection method. The principle of detection is based on the reduction or oxidation of CATS in an electrical cell. After the separation, analytes in the column eluant pass over an electrode and are oxidized or reduced by the potential of the electrode. When the potential of the working electrode versus the reference electrode is positive and greater than the potential required to electrolyze the analyte molecules, the molecules are oxidized; with negative potential the molecules are reduced. The electrons released from or captured by the analytes can be measured as electrical current that is proportional to the amount of analytes. The working electrode can be gold, silver, platinum, carbon paste and glassy carbon. Principle and design of electrochemical detectors has been reviewed in detail (Nyyssönen & Parviainen 1989).

Depending on the design of the analytical cell, there are 2 main types of detectors: amperometric and coulometric. Amperometric cells are usually of thin-layer structure and due to the cell geometry only 10% of the analyte molecules undergo electrolysis. On the other hand, the coulometric detectors consist of fully porous graphite working electrode to provide large surface area for efficient electrochemical reaction. Nearly all analyte molecules complete the reaction. Thus, the coulometric type is many folds more sensitive than the amperometric type. Nevertheless, the amperometric detector is cheaper and sensitive enough for measurement of urinary CATS. For ion-pair reversed phase HPLC system, it is common to find a positive potential of around 0.70 mV using amperometric detectors (Oka *et al* 1982, Wu & Gornet 1985, Fosti *et al* 1987, Anderson *et al* 1988). Negative potentials were used with more complicated coulometric types (Hugh *et al* 1987, Weinkove 1991).

CONCLUDING REMARKS

The ion-pair reversed phase HPLC method is the most popular and robust method for routine measurement of urinary CATS. It provides sufficient sensitivity and specificity and is robust to be adaptable onto different HPLC instrumentation. However, the preanalytical factors such as preservatives for urine and the pretreatment method before HPLC measurement need systematic study.

II. METHOD OF MEASUREMENT IN THIS STUDY

PRINCIPLE OF THE METHOD

An ion-pair reversed phase HPLC method was developed. A published mobile phase using acetate-citric acid buffer and octanesulfonate as the ion-pair reagent was modified (Weicker *et al* 1984). The percentage of methanol used in the mobile phase was increased to improve the separation time because of the use of a different stationary phase. Electrochemical detector (ECD) was used for the quantitation of separated CATS.

An acid preservative, 0.5 mM HCl, is used in this project and this was selected after a systematic study on the effect of acid concentrations and storage temperatures. A published alumina extraction method for urinary CATS was adapted (Davidson & Fitzpatrick 1985). This method used a 3 M TRIS buffer to maintain an optimal pH condition during the adsorption process to overcome apparent interference from high urinary creatinine. Furthermore, the eluted CATS from the alumina was further washed with ethyl acetate to remove electro-active species from the eluant. This enabled low noise-to-signal chromatograms suitable for accurate quantitation.

MATERIAL AND METHODS

Material

Adrenaline-barbiturate (A), noradrenaline-L-tartrate.H_2O (NA), 3,4-dihydroxybenzylamine hydrobromide (DHBA), 3-hydroxytyramine (dopamine, DA), alumina (grade I), ascorbic acid, Trizma, ethylenediamine-tetraacetic acid disodium salt (EDTA), anhydrous sodium acetate, anhydrous citric acid, and 1-octanesulfonic acid sodium salt were obtained from Sigma Chemical Company (St. Louise, MO, USA). Methanol and ethyl acetate (HPLC grade) and triethylamine (AR grade) were purchased from Merck (Darmstadt, Germany). Sodium metabisulphite was obtained from British Drug House Chemical Ltd. (England). Deionized water was prepared by the Milli-Q water system from Waters Company (Millipore, Milford, MA, USA).

Equipment

Solvent delivery pump system model 600E, Nova Pak octadecylsilane column (4 μm particle size) with radial compression module (RCM) module and guard column, autosampler WISP model 712, electrochemical detector (ECD) model 460, and the PowerLine software on NEC Powermate PC for system control and data integration were from Waters Company (Millipore, Milford, MA, USA).

Reagent preparation

Stock standard solutions

Stock NA, A, DA, and DHBA standard solutions of 500 μmol/l were prepared by weighing the appropriate amount of the chemicals, dissolved in 0.1 M HCl, and made up to volume with 0.1 M HCl in volumetric flasks. The stock solutions were stable at 4 °C for 6 months and at -20 °C for over 2 years.

Working standard solutions

Three working standard solutions were prepared by adding appropriate volumes of the stock standard solutions to 100-ml volumetric flasks and made up to volume with 0.1 M HCl solution. The final concentrations of CATS were: 200 nM NA, 50 nM A and 500 nM DA in standard 1; 400 nM NA, 100 nM A, and 1000 nM DA in

standard 2; and 800 nM NA, 150 nM A and 2000 nM DA in standard 3. These standard solutions were aliquoted and kept at 4°C for 1 month or stored at -20°C for longer period.

Internal standard solution

The internal standard solution was prepared by adding 400 µl of the DHBA stock standard solution into a 100-ml volumetric flask and made up to volume with 0.1 M HCl. The resulting concentration was 2 µM. This solution were stable at 4 °C for at least 1 month.

3M Tris buffer

This buffer was prepared by weighing 363 g of Trizma and 50 g EDTA in a 1-liter flask and deionized water was added to make up to about 800 ml. The solution was mixed constantly on a magnetic stirrer kept at 40 °C until the solution was clear. The pH was adjusted to 8.75 with concentrated HCl and the solution was made up to 1 liter in a volumetric flask with deionized water. This buffer was kept at 4 °C.

0.1M Tris buffer

This buffer was prepared by diluting the 3M Tris buffer 30 times with deionized water and kept at 4°C.

Reducing solution

This solution was prepared fresh prior to use by dissolving 0.19 g sodium metabisulphite and 0.175 g ascorbic acid in 10 ml deionized water. This solution was stable for 24 hours.

Alumina

The alumina was pretreated according to the published method (Aston & Sayre 1962). Alumina was washed sequentially with different concentrations of Hcl at different temperatures. The acid was washed away with deionized water and the alumina was finally dried thoroughly in an oven.

Stock mobile phase

The stock mobile phase contained 1 M sodium acetate, 0.4 M citric acid and 2 mM of EDTA. This was prepared by dissolving 82.03 g anhydrous sodium acetate, 76.84 g anhydrous citric acid, and 0.7445 g of EDTA disodium salt.$2H_2O$ in deionized water and made up to 1 litre in a volumetric flask.

Working mobile phase

The working mobile phase contained 1/20 dilution of the stock mobile phase, 2 mM of octanesulphonate, 1 mM of triethylamine, and 14% methanol. This was prepared by adding 50 ml of the stock mobile phase, 0.4326 g of sodium-1-octane sulfonate, 139 µl of triethylamine and 140 ml of HPLC grade methanol to a 1-litre volumetric flask and made up to volume with deionized water. The working mobile phase was allowed to stand overnight before filtering with 0.45 micron aqueous filter, otherwise, the filter would get blocked easily.

Preservation of urine samples

For the collection of 24-h urine from human subjects, 100 ml of 0.5 M HCl solution was added to each 2-litre plastic container. The urine samples were kept at 4 °C if the analyses could not be done within 3 days. Otherwise, the samples were frozen at -20 °C and the analyses were completed within one month.

For the collection of 24-h urine from rats, 10 ml of 0.5 M HCl solution was added to each plastic urine container of the metabolic cage. Storage conditions for the sample were similar to that for human samples.

Alumina extraction of urinary free CATS

The extraction procedure was modified from a published method (Davidson & Fitzpatrick 1985). Two steps of the method were modified. Firstly, after the adsorption of free CATS onto the alumina in 3 M Tris buffer, the alumina was washed with 0.1 M Tris buffer instead of 0.1 M barbitone buffer. Secondly, the adsorbed free CATS were eluted with 0.1 M HCl instead of 0.5 M HCl.

A. The following solutions were added to a screw capped glass tube:

5 ml urine/standards/QC
+
250 µl reducing solution
+
1 ml internal standard solution
+
5 ml 3M Tris buffer
+
100 mg treated alumina.

B. The glass tube was tumble-mixed on a roller mixer for 20 min.

C. The tube was centrifuged for 3 min at 800 g.

D. The supernatant was carefully discarded without removing the alumina.

E. 10 ml of 0.1M Tris buffer was added and the tube was tumble-mixed for 5min.

F. The tube was centrifuged for 3 min at 800 g.

G. The supernatant was carefully discarded without removing alumina.

H. 1 ml of 0.1M HCl was added and the mixture was vortexed for 30 sec.

I. The tube was centrifuged for 3 min at 800 g.

J. The supernatant was transferred to an Eppendorf centrifuge tube.

K. The Eppendorf centrifuge tube was centrifuged for 5 min at 16000 g.

M. 900 µl of the supernatant was pipetted to 8 ml ethyl acetate in a glass centrifuge tube and vortexed for 1 min.

N. The glass tube was centrifuged for 3 min at 800 g.

O. The upper organic layer was discarded and the lower aqueous layer was transferred into a sample vial for HPLC analysis.

Separation and quantitation of free CATS by HPLC

A. The working mobile phase was delivered at a flow rate of 2 ml/min by the E600 pump. Helium was sparged at 30 ml/min continuously for degassing. The used mobile phase was recycled after filtering with 0.45 µm filter until the background current exceeded 10 nA.

B. The NOVAPAK C18 (4µm particle) was used with RCM and guard column.

C. Applied potential on the ECD was set at +0.60 V.

D. Analysis time per sample was programmed for 16 min.

E. 15 µl of sample was injected by the autosampler WISP.

F. Powerline programming for signal collection and integration was as follows:

1.	signal collection interval		1 second
2.	time range		4 - 16 min
3.	peak integration:	width	0.1 min
		height	0.005 nA
		slope	0.001 nA/min
		minimum area	0.0001 nA.min
		smoothing	5 points

G. Identification of the CATS was achieved by the retention times.

H. Standardization was based on the standard analyte to internal standard peak area ratios.

I. Two quality control samples, prepared by spiking of 2 sets of pure standards into acidified human urine pool, were included into each batch of analysis.

J. Quantitation of unknown samples was performed by comparing the analyte to internal standard peak area ratios on the Powerline workstation.

Statistical analyses

Statistical analyses were performed using the WinSTAR™ program (Anderson-Bell, Arvada, CO, USA) on personal computer. Linear regression method was used to assess the linearity of standard curves. Analysis of variance (ANOVA) was used for multiple comparisons of sample means. The pairs with significant difference were identified with Scheffe test and also by Student's paired-t test. Significance level was set to $p<0.05$ for all these methods.

RESULTS

Optimization of the method

Optimization of mobile phase for the separation of CATS by HPLC

According to the method published by Weickers *et al* (1985), the mobile phase contained 5% methanol. It was able to separate the 3 free CATS and the internal standard within 15 min at a flow rate of 1.0 ml/min. However, due to the use of a different type of column in the present system, it took more than an hour to complete the separation using the same mobile phase and the same flow rate. Optimization of the system was attempted by modifying the composition of the mobile phase and the flow rate. The aim was to obtain base line separation of the 3 free CATS and the internal standard in 15 min similar to the original method.

An aqueous standard solution, containing 2.5 μM of NA, A, DHBA and DA, was prepared by appropriate dilution of the individual CATS stock standard solutions with 0.1 M HCl solution in a volumetric flask. Three working mobile phase solutions were prepared as described in **Reagent preparation**. Each solution contained different amounts of methanol: 7%, 10% and 14%. The mobile phase containing 7% methanol was used to separate the different analytes in the aqueous standard solution as described in **Separation and quantitation of free CATS by HPLC** except that the flow rate was programmed at 1.0 ml/min. The time required to achieve base line separation of all the analytes was recorded. The procedure was then repeated using the mobile phase containing 10% methanol at a flow rate of 1.5 ml/min. Finally, the 14% methanol mobile phase was repeated similarly at flow rates of 1.5 and 2.0 ml/min.

The variation of retention times under different conditions is summarized in Table 3-3. By increasing the flow rate and methanol concentration in the mobile phase, baseline separation of the 4 analytes in the aqueous standard was achieved within 15 min. Methanol and flow rate affected each analyte differently under various conditions. With the 14% methanol mobile phase and a flow rate of 2.0 ml/min, the retention

times for each analyte over a period of 72 h were recorded (n=14). A typical chromatogram is shown in Figure 3-2. The coefficients of variation for the retention times of all analytes were less than 1%. Thus, the optimized conditions for the HPLC was consistent and reliable for the subsequent studies.

Optimization of the applied potential on the ECD

The aqueous standard solution prepared for optimization of the mobile phase was used. Chromatographic separation of the analytes was performed in duplicate as previously described. Applied potential on the ECD was set to +0.30 V. The working potential was slightly different from the set value and was recorded. Retention times, peak heights and peak areas of each analytes were recorded.

The experiment was then repeated with applied potentials of +0.35 V, +0.40 V, +0.50 V, +0.60 V, +0.70 V, and +0.80 V. A minimum of 4 h was allowed for a new applied potential to become stable on the ECD before the aqueous standard solution was injected. The peak areas and peak heights of each analyte were plotted against the working potential to construct the hydrodynamic voltammograms for each analyte.

Changes of peak heights and peak areas of the different analytes with different applied potentials are shown on Figures 3-3. The peak heights and areas increased sharply from +0.30 V to +0.45 V, but became stabilized after +0.55 V. The pattern of these hydrodynamic voltammograms was similar for all the 4 analytes. The potential of +0.60 V was chosen for all subsequent studies. Higher potential was not desirable as more electroactive species in the samples and the mobile phase would be oxidized. This could increase the background current significantly especially when the mobile phase is recycled.

Table 3-3: The effect of methanol concentration and flow rate on elution time of CATS

% Methanol	Flow rate (ml/min)	Total elution time (min)
7	1.0	60
10	1.5	30
14	1.5	25
14	2.0	15

Results of the elution time are given as the mean of triplicate analyses.

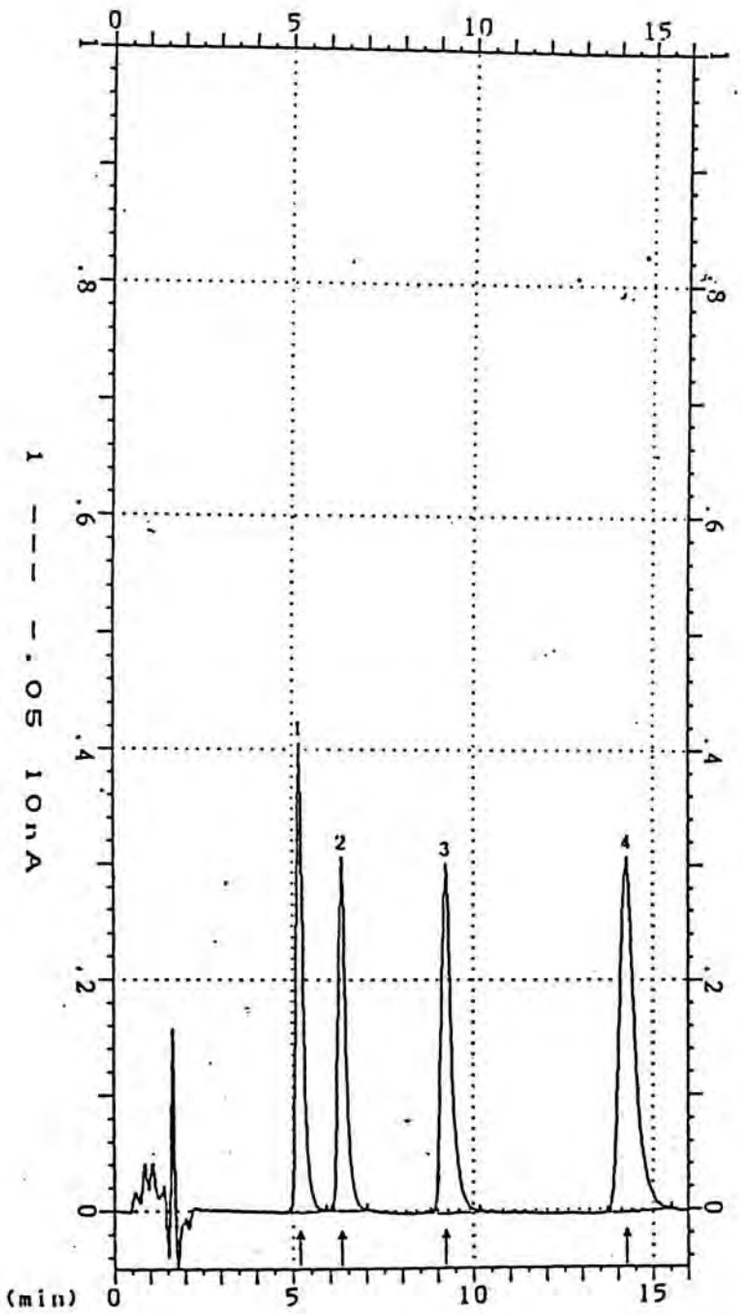


Figure 3-2: **A typical chromatogram for the separation CATS by the developed HPLC method.**

1 Noradrenaline **2 Adrenaline**
3 DHBA (internal standard) **4 Dopamine**

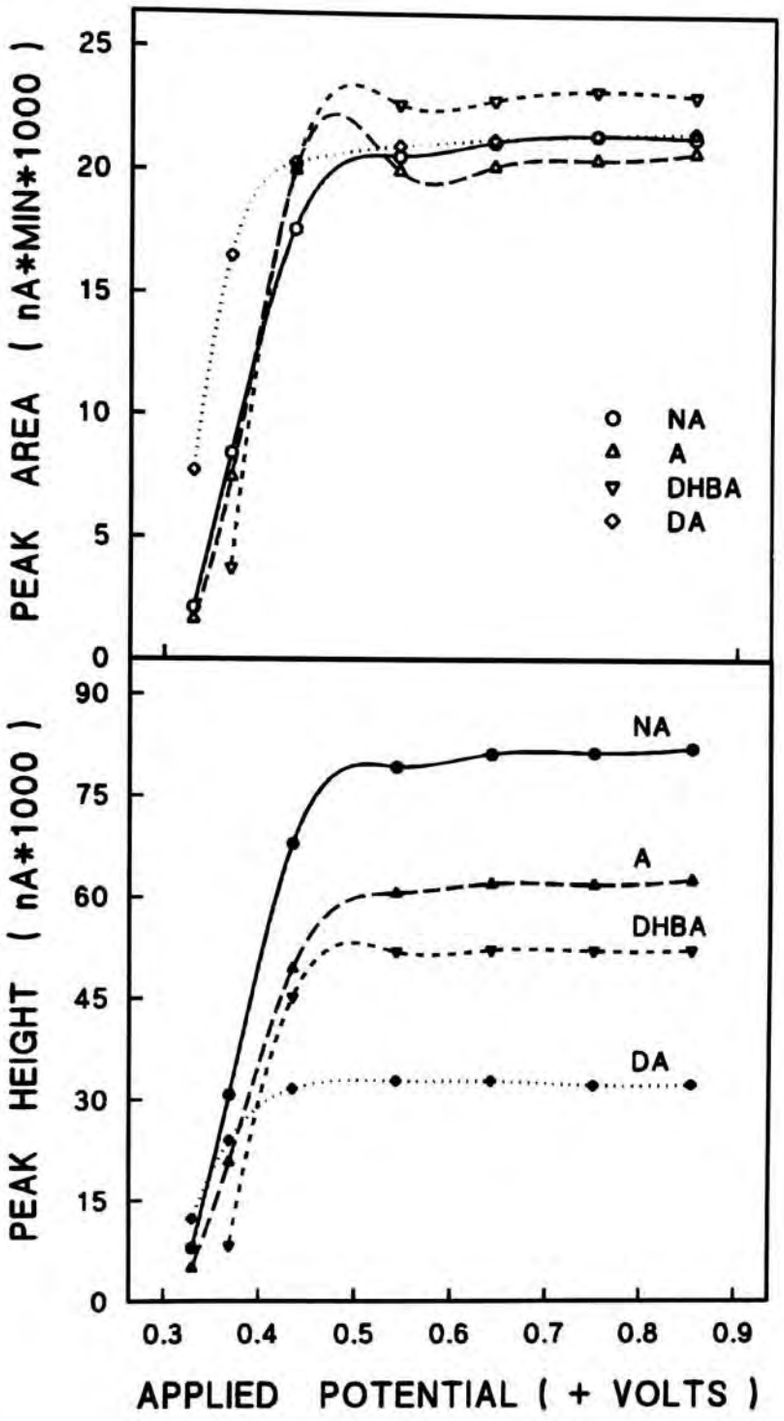


Figure 3-3: Hydrodynamic voltammograms for NA, A, DA and DHBA.

Linearity range and detection for the ECD

A series of 28 working standard solutions containing NA, A and DA were prepared by appropriate dilution of the stock standard solutions in volumetric flasks and made up to the volume with 0.1 M HCl. Concentration of the 3 analytes in each working standard solution was the same, ranging from 5 nM to 5000 nM. Standards were subjected to HPLC analysis in duplicate as previously described. Peak areas of each analyte were recorded. The mean peak areas were plotted against the expected concentration to determine the linearity range of the ECD. The lowest detection limit for the detector was determined by the concentration of analyte that could not be integrated by the system.

The linearity curve for DA is shown on Figures 3-4. ECD response was linear for the standard solution containing 30 nM to 5000 nM. There was no peaks that could be integrated for standard solutions less than 30 nM. The injection volume for each standard solution was 15 μl. Thus, the lowest detection limit for DA was 0.45 pmole and the linearity was at least up to 75 pmoles. Linearity curves for NA and A standards were similar to those of DA standards.

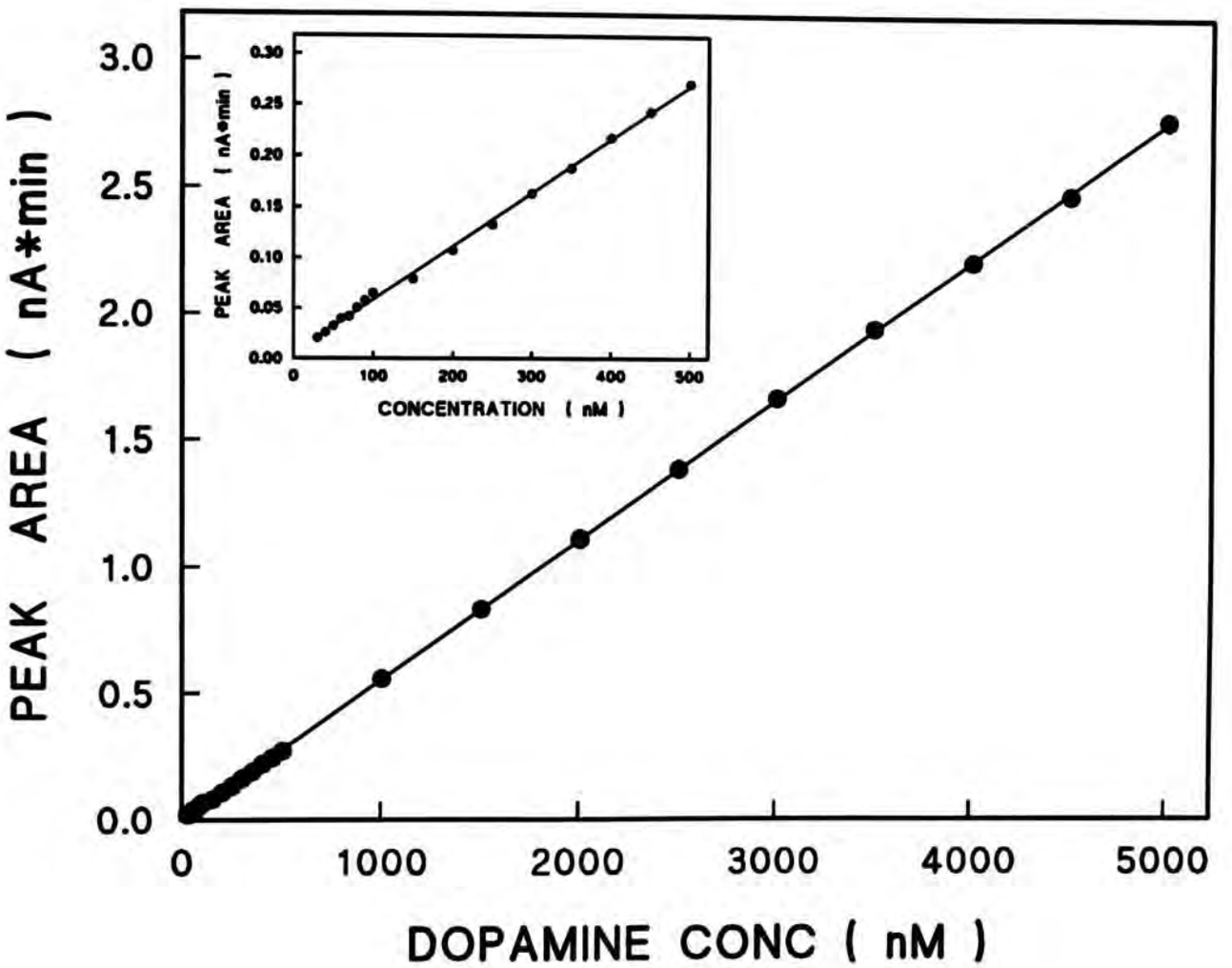


Figure 3-4: Linear range of DA on the ECD

For concentration ranging from 30 - 5000 nM, the regression equation:
$y = 5.53 \times 10^{-4} + 0.00166$ $r = 0.9999$
For concentration ranging from 30 - 500 nM, the regression equation:
$y = 5.27 \times 10^{-4} + 0.00632$ $r = 0.9992$

Effect of the amount of alumina used on quantitation

The extraction of urinary CATS with alumina was adapted from a published method (Davidson & Fitzpatrick 1985). The original article stated that 50 mg to 350 mg per 10 ml of urine sample produced no significant change in the recovery. When the method was adapted, it was found that ascorbic acid was eluted at 1 min after injection. However, the total elution time for the ascorbic acid peak was inconsistent. There were situations where the NA, A and DHBA peaks were eluted with the ascorbic acid peak causing problem for automatic peak integration which in turn affected the quantitation. Preliminary experiments indicated that the amount of alumina could affect the amount of ascorbic acid present in the final solution injected into the HPLC. The higher ascorbic acid concentration in the sample would take a longer time for elution. To minimize the interference of ascorbic acid on peak integration, the amount of alumina used was optimized.

The aqueous working standard solution prepared for the optimization of the mobile phase was extracted with 100 mg of alumina as previously described. Ethyl acetate washing was not done. Chromatographic separation of the analytes was performed as previously described. The total elution time for the ascorbic acid peak was recorded. The experiment was then repeated using 200 mg and 300 mg of alumina.

Total elution time of the ascorbic acid peak for 100 mg of alumina was 4.5 min and did not interfere with the NA peak on the chromatogram. Elution times of ascorbic acid peak for the 200 mg and 300 mg alumina were 7 min and 10 min, respectively. These elution times interfered with the integration of NA and A. It was therefore decided to use 100 mg of alumina to the extraction mixture. A tailor-made plastic cup was made to weigh the alumina for 100 mg ± 5%.

Effect of acid solutions on the elution of CATS from alumina

The effect of different acid solutions on the elution of adsorbed CATS from the alumina was examined using the aqueous standard solution described above. Extraction of free CATS from the standard solution was performed as previously described. Elution of the adsorbed CATS was performed using 5 ml of 0.5 M acetic

acid in duplicates. Ethyl acetate washing was excluded from the procedure. Chromatographic separation and quantitation of free CATS were as previously described. The experiment was then repeated using 0.5 M HCl and 0.1 M HCl. An aqueous standard solution was also subjected to the chromatographic procedure to obtain the expected peak area before alumina extraction. The % recovery for each analyte after the different acid elutions from the alumina was calculated by comparing the peak areas with that without the alumina extraction.

The results are summarized on Table 3-4. 0.1 M HCl gave the highest % recovery and was chosen to be the acid for elution in the subsequent studies. Furthermore, the use of a dilute acid for sample preparation would be desirable to provide a better condition for long-term stability of the HPLC column.

Table 3-4: Effect of acid solutions on elution of CATS from alumina

	% Recovery			
Acid solutions	NA	A	DHBA	DA
0.5 M acetic acid	96.4	96.1	97.7	99.2
0.5 M HCl	95.5	98.6	98.3	97.0
0.1 M HCl	101.1	103.9	103.6	103.1

Results are given as the mean of triplicate analyses.

Analytical performance of the method developed

Linearity of the method

A series of 10 aqueous CATS standard solutions was prepared by appropriate dilutions of the stock standard solutions in volumetric flasks and made up to volume with 0.1 M HCl. The concentration of the NA, A and DA in each standard was the same. The range of concentrations was from 50 nM to 10 μM. The standard solutions were extracted with alumina and HPLC separation of the CATS was as described before. However, the injection volume had to be adjusted to keep the peak heights in the range of the detector's scale. The smallest volume used was 2 μl for the highest standard solution. Ratios of peak areas between the analytes and the internal standard were calculated and plotted against the expected concentrations.

The linearity curve for DA standards are shown on Figure 3-5. The developed HPLC method could measure DA up to 10 μM in concentration. Linearity curves for NA and A were similar to that of DA. However, the injection volume had to be lower for concentrations higher than 2.5 μM. This was more convenient compared to the alternative of diluting the sample and then repeating the entire procedure. Furthermore, urinary CATS concentration higher than 2.5 μM is not common.

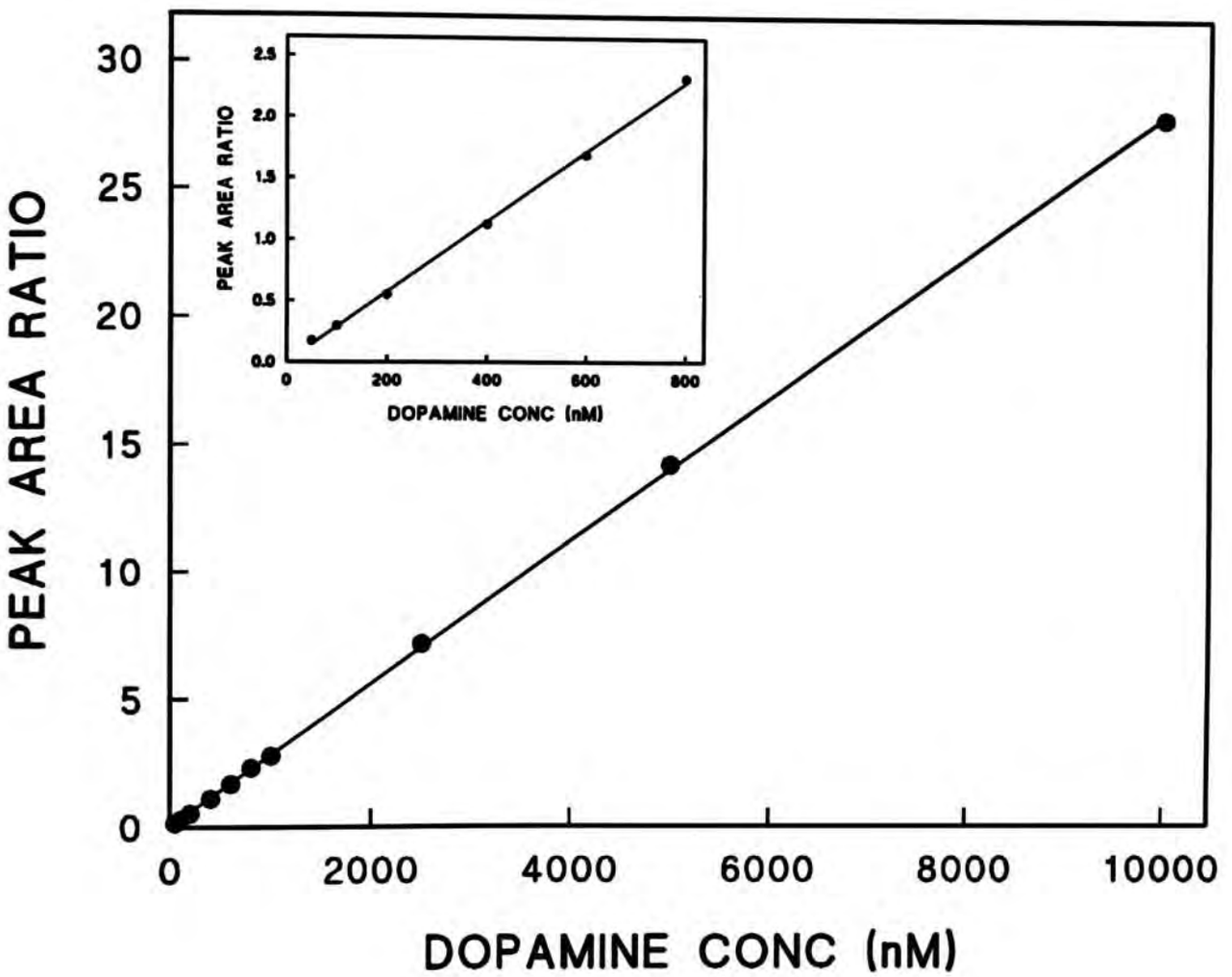


Figure 3-5: Linear range of DA of the HPLC-ECD method

Peak area ratio = Peak area of DA ÷ Peak area of DHBA
For concentration ranging from 50-10000 nM, the regression equation:
$y = 2.80 \times 10^{-3} + 0.0538$ $r = 0.9999$
For concentration ranging from 50 - 800 nM, the regression equation:
$y = 2.86 \times 10^{-3} + 0.00479$ $r = 0.9998$

Intra-batch precision performance and recovery study using spiked human urine samples

An acidified 24-h urine was obtained from the routine laboratory of this Department at the Prince of Wales Hospital. Three working standard solutions containing different amounts of NA, A and DA were prepared by appropriate dilutions of the stock standard solutions with 0.1 M HCl in volumetric flasks. Final concentrations of the 3 standard solutions were 2 μM NA, 1 μM A, 5 μM DA for solution 1; 4 μM NA, 2 μM A, 10 μM DA for solution 2; and 6 μM NA, 3 μM A, 20 μM DA for solution 3.

One milliliter of 0.1 M HCl was added to a 10-ml volumetric flask and made up to the volume with the acidified urine. The procedure was then repeated replacing the 0.1 M HCl with the 3 standard solutions. These 4 urine samples were subjected to alumina extraction and HPLC analysis in triplicates as previously described. The % recovery was calculated for the urine samples containing the spiked standards. Intra-batch precision performance was determined by calculating the CV values among the triplicates.

The results of recovery and intra-batch study are shown on Table 3-5. For NA, the average % recovery was 100.8% (range 99.5-102.8%). The intra-batch precision (CV) ranged from 1.4 to 3.2%, depending on the concentration. For A, the average % recovery was 93%, (range 89-96%). The intra-batch precision ranged from 3.2 to 17.4%. The precision performance was poor at the low end of the concentration range. Finally for DA, the average % recovery was 98.5%, (94-101.5%). The intra-batch precision CV% ranged from 1.0 to 3.1%.

Table 3-5: Results of recovery and intra-batch precision study

	Concentration of Standard (nM)	Measured Concentration (nM)	% Recovery	Intra-batch % CV
NA				
	0	304	/	3.2
	200	503	99.5	3.0
	400	715	102.8	2.2
	600	905	100.2	1.4
A				
	0	58	/	17.4
	100	147	89.0	3.6
	200	246	94.0	8.4
	300	346	96.0	3.2
DA				
	0	1002	/	1.0
	500	1472	94.0	1.0
	1000	2003	100.1	3.1
	2000	3031	101.5	1.1

Inter-batch precision performance

Two acidified 24-h urine samples were obtained from the routine laboratory. They were aliquoted into 5-ml portions and stored at -20 °C and served as quality control samples. For every batch of analysis, two quality control samples were thawed and analyzed. Results of the inter-batch precision performance are shown on Table 3-6. Due to the low concentration of A in both samples, the CV was higher. The performance was expected to be similar to those of NA and DA which were present in higher concentrations. For both NA and DA, the CV were less than 10%.

Table 3-6: Inter-batch precision performance using 2 quality control urine samples

Quality control Sample	CATS	Mean Concentration (nM)	C V %
A (n = 97)	NA	279	2.0
	A	69	11.6
	DA	1011	4.8
B (n = 47)	NA	91	9.9
	A	27	29.6
	DA	612	6.2

Stability studies of urinary CATS under different storage conditions

Effects of acid concentration on aqueous CATS standards

The use of different acid concentrations to preserve urine samples could affect the efficiency of alumina extraction. The optimal pH of 8.6 required for the extraction is usually maintained by buffer solutions. Excess acid could affect the buffering capacity resulting in a lower pH environment. Furthermore, an increase in inorganic acid concentration could enhance the oxidative destruction of CATS in the absence of reducing solutions.

A series of 5 CATS standard solutions, concentrations ranging from 100 to 1000 nM, was prepared by appropriate dilution of stock standard solutions in volumetric flasks and made up to volume with 0.1 M HCl. Standard solutions were allowed to stand at room temperature (23 °C) for 2 h. Then, alumina extraction and HPLC analysis were performed as previously described. The peak area ratios of each analyte with the internal standard were calculated. Another 4 experiments were similarly performed using 4 different series of standard solutions that were made with 0.25 M, 0.50 M, 1.0 M and 2.0 M HCl. The effect of acid concentration on the peak area ratios for each analyte was assessed by 1-way ANOVA with replications.

The 2M acid solution overcame the buffering capacity of the 3 M Tris buffer used in the extraction mixture. The pH value of the extraction mixture was around 6.0 measured by pH paper. Results for this series of standard solutions were not included in the statistical analysis. For the others, pH values of the extraction mixtures were close to 8.5 and the variation in results could not be due to suboptimal pH for alumina extraction. The acid concentration appeared to have significant effect for NA and DA ($p=0.0125$ and 0.0130, respectively) and for A the effect was not significant ($p=0.0510$). The peak area ratios of the analytes decreased with increasing acid concentration. The concentration of CATS in 1.0 M HCl solutions were significantly different from those of 0.1 M HCl solutions. The relative changes between the results of these 2 acid solutions were - 1.0% for NA, -2.1% for A and -3.7% for DA. These data suggest that the use of strong acid solutions denatured the free CATS. It also

indicated that DA is more susceptible to this oxidative destruction than the other 2 CATS.

Effects of acid concentration and storage temperature on human urine free DA

The effect of different acid concentrations to preserve urinary free CATS has not been well documented. It has been commented that the use of high acid concentration could lead to the hydrolysis of conjugated CATS giving falsely high free CATS concentrations in the urine samples (Euler & Von Lishajko 1961, Henry & Pratt 1990). Furthermore, the process of hydrolysis is affected by temperature. An increase in temperature can enhance the rate of hydrolysis. Thus, this experiment was designed to investigate the effect of acid concentration at different storage temperatures on urinary free DA.

Fresh urine samples were collected from healthy individuals and pooled. Twenty-five milliliters of deionized water were added to a 250-ml volumetric flask and made up to volume with the urine pool. This represented the urine sample without any acid preservative. The preparation of acidified urine samples was similarly performed using 25 ml of 1 M, 2.5 M, 5 M, 7.5 M and 10 M HCl solutions. Thus, a series of acidified urine samples containing 0.1 M, 0.25 M, 0.50 M, 0.75 M and 1 M HCl was produced. Each urine sample was aliquoted into 10-ml plastic tubes and stored at different temperatures. Three sets were kept in a 37 °C water bath; 3 sets were kept in the dark at room temperature (23 °C); and another 7 sets were kept at 4 °C in the cold room.

Six hours after the preparation of the acidified urines, one set of urine samples was removed from the storage locations and extracted and analysed as described before. This was then repeated at 24 h and 48 h for the 3 storage temperatures. For the samples stored at 4 °C, the remaining 4 sets were measured at 1, 2, 3 and 4 weeks time.

Results are shown on Figure 3-6. The results show that urinary free DA increased with increasing acid concentrations and at higher temperature, probably due to the

hydrolysis of conjugated DA in the sample. The urinary free DA increased 200 % when kept in 1 M HCl at 37 °C after 24 h and 300 % after 48 h. The previous experiment also indicated that DA was susceptible to oxidative destruction at high acid concentration. Thus, the actual rate of hydrolysis could be higher. On the other hand, the absence of acid preservative caused a 4% decrease in free DA at 4 °C after 48 h. Since the between-batch precision performance exceeded 4 %, this magnitude of change would not be considered significant. A final acid concentration of 0.1 and 0.25 M at 4 °C was sufficient to protect urinary free DA for about 1 month. A detailed study was then designed to investigate the effects of storage conditions for all other urinary free CATS.

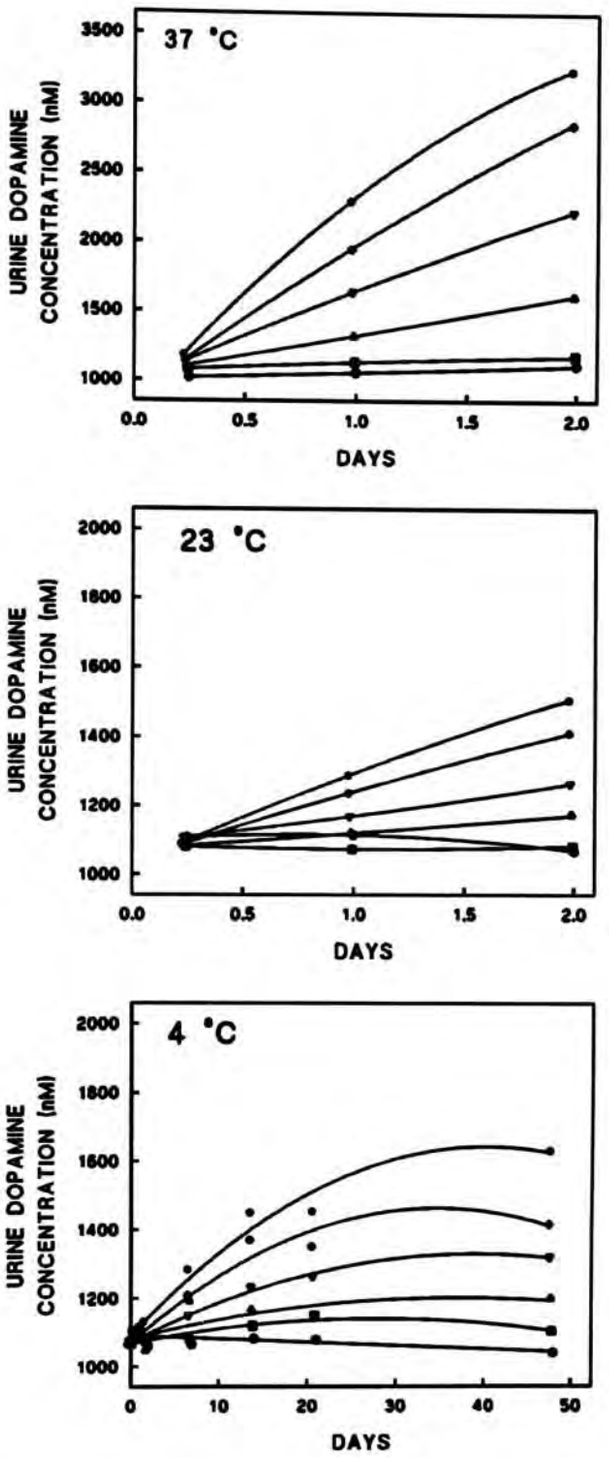


Figure 3-6: Effects of acid concentration and storage temperature on human urinary free DA

- ● 0.0 M HCl
- ■ 0.10 M HCl
- ▲ 0.25 M HCl
- ▼ 0.50 M HCl
- ◆ 0.75 M HCl
- ★ 1.0 M HCl

Effects of storage temperature and duration of alumina extracts of human urinary free CATS

HPLC analysis is a sequential process. Extracted free CATS are stored in 1-ml glass sample vials and can be inside the autosampler up to 25 h at room temperature. Therefore, an experiment was designed to study the effect of storing extracts of free CATS inside the autosampler (the WISP) over a period of 36 h.

One fresh human spot urine sample was collected from a healthy volunteer. The sample was separated into 12 aliquots. The alumina extraction and HPLC analysis were as described previously. After completion of the initial HPLC analysis, the sample vials were kept inside the WISP and measurements were repeated at 12 h, 24 h and 36 h. Results were analyzed by 1-way ANOVA to study the effect of storage duration inside the WISP.

The results are summarized in Table 3-7. Significantly higher NA results were observed after 24 h and 36 h ($p<0.001$). The mean NA for both 24-h and 36-h measurement was 3.7 % higher than the 0-h measurement. Although this was statistically significant, the magnitude of change was still within the analytical imprecision performance. There were no significant effect for A and DA ($p=0.484$ and 0.140, respectively). Thus, keeping the extracts inside the WISP for 36 h would not affect the results of DA.

Unexpected breakdown of the HPLC system might take over a week's time for repair and extracted samples may have to be stored until the problem is resolved. A batch of 12 acidified human urine samples was extracted. After the first measurement, the sample vials were kept in the dark inside the cold room at 4 °C for 1 week and 2 weeks. They were taken out and the measurement was repeated at the end of the storage period. Statistical analyses were the same as described above.

The results are summarized on Table 3-8. Duration of storage had a significant effect on both NA and A ($p<0.001$ and $p=0.003$, respectively). After 1 week and 2 weeks, the NA concentration increased by 7.2% and 6.8 %, respectively. For free A, there

was a significant decrease of 23.4% after storage for 2 weeks. There was no significant change for free DA ($p=0.363$) stored at 4 °C over 2 weeks. It was also noticed that the ascorbic acid peak at the solvent front decreased in size with storage. Thus, extracted samples should be analyzed within 1 week to avoid significant changes of NA and A, but the DA remained unaffected.

Table 3-7: Effects of storage over 36 h at room temperature on extracts of urinary free CATS.

Time (h)	Concentration (nM) Mean ± S.D.			Relative % Change		
	NA	Aa	DA	NA	A	DA
0	273±15.5	53±11.2	1052±86.4	/	/	/
12	277±16.7	60±11.2	1064±81.7	+ 1.5	+ 13.2	+ 1.1
24	283±15.6*	58±13.3	1069±69.8	+ 3.7	+ 11.3	+ 1.6
36	283±15.2*	59±13.5	1083±72.2	+ 3.7	+ 11.3	+ 2.9

The changes of mean concentrations were compared with the 0-h measurement using Scheffe test (* for $p<0.002$).

Table 3-8: Effects of storage over 2 week at 4 °C in the dark on extracts of urinary free CATS.

Time (wk)	Concentration (nM) Mean ± S.D.			Relative % Change		
	NA	A	DA	NA	A	DA
0	221±43.0	64±26.7	1275±282.9	/	/	/
1	237±38.5**	59±22.1	1280±279.6	+ 7.2	- 7.8	+ 0.4
2	236±37.5**	49±12.5*	1280±289.0	+ 6.8	- 23.4	+ 0.4

The changes of mean concentrations were compared with the 0-wk measurement using Scheffe test (* for $p<0.005$; ** for $p<0.0001$).

Effects of storage duration on acidified human urinary free CATS

To simulate the collection and handling of human urine samples, the effects of storage duration on acidified human urinary free CATS were studied. A final acid concentration of 0.025 M HCl was chosen based on the previous experiment. The summer ambient temperature in Hong Kong is often over 30 °C. To simulate the collection of 24-h urine samples by outpatients, acidified samples were stored at 30 ° C for 24 h and 48 h. For cross-sectional studies, urine samples are usually stored frozen and the analysis performed in big batches to improve the precision performance. To study the effects of storage, acidified urine samples were stored at -20 °C for over 3 months.

A batch of 10 fresh random spot urine samples was collected from healthy laboratory staff. Each sample was separated into seven 5-ml aliquots and stored in plastic tubes. To each tube, 500 µl of 0.25 M HCl was added. One tube was immediately extracted and analyzed and two tubes were stored in a 30 °C water bath and the urine samples were similarly processed and analysed 24 and 48 h later. The other 4 tubes were stored frozen at -20 °C. These samples were thawed, processed and analyzed at 2 weeks, 1 month, 2 month and 3 months later. The effects of storage duration on the 3 CATS were analyzed by 1-way ANOVA with replications as previously described.

The results are summarized in Table 3-9. For samples stored at 30 °C, there were no significant changes for NA and A among the 0, 24-h and 48-h periods ($p=0.743$ and $p=0.0929$, respectively) but DA was affected by storage duration ($p=0.0180$). DA increased by 3.9% after 24 h, but showed no significant difference after 48 h. This could be caused by a systematic drift of the assay for the 24-h samples. Thus, acidified urine samples could be stored for 48 h even at 30 °C. For frozen samples at -20 °C, the ANOVA test showed that both NA and A were affected ($p=0.0004$ and $p=0.0118$, respectively). Follow-up with the Scheffe test, it showed that NA could be stored for up to 1 month and A for 2 months. No significant difference was found for DA results ($p=0.115$). The concentration decreased by 5.5% after one month, but then increased by 4%. This magnitude of changes was acceptable as the between-

Table 3-9: Effects of storing human urine samples at 30 °C and -20 °C on free CATS.

Storage Conditions	Concentration (nM) Mean ± S.D.			Relative % Change w.r.t. 0-h sample		
	NA	A	DA	NA	A	DA
Fresh Samples	147±77.1	97±53.0	1286±543.8	/	/	/
24 h at 30 °C	146±81.8	96±51.1	1337±593.0	- 0.7	- 1.0	+ 3.9
48 h at 30 °C	148±81.1	91±51.6	1298 ±587.7	+ 0.7	- 6.2	+ 0.9
2 weeks at -20 °C	149±78.0	95±54.9	1235±537.0	+ 1.4	- 2.1	- 4.0
1 month at -20 °C	159±90.6	94±51.1	1215±404.8	+ 8.2	- 3.1	- 5.5
2 months at -20 °C	167±90.8*	101±52.3	1341±566.7	+ 13.6	+ 4.1	+ 4.3
3 months at -20 °C	161±90.4	83±48.0	1343±607.6	+ 9.5	- 14.4	+ 4.4

The changes of mean concentrations were compared with the fresh samples using Scheffe test (* for $p<0.005$).

batch precision CV was about 6 - 7%. Thus, samples could be stored frozen at -20°C for up to one month after the collection.

Effects of acid concentration and storage duration on rat urinary free CATS

Composition of rat urine samples is different from that of human urine. For example, rat urine samples contained a higher bicarbonate concentration than that of human. The amount of acid required to preserve human urine sample might not be enough for rat urine sample because the bicarbonate can neutralize the acid, thus affecting the stability of urinary free CATS. An experiment was set up to study the effects of acid concentration and storage duration on rat urinary free CATS at room temperature over a period of 24 h and 48 h.

Twelve rats were isolated in metabolic cages for the collection of 24-h urine samples. Ten milliliters of 0.1 M HCl was placed in the urine collection container to provide the initial preservative. After the urine volumes were recorded, samples with a volume greater than 20 ml were regarded as a complete sample. Those samples with less volume were pooled to make up a volume of more than 20 ml and then used as a complete sample.

Each complete sample was separated into nine 2-ml aliquots. The first aliquot ("0-h"sample) was immediately extracted. The remaining 8 aliquots were acidified in duplicates using 0.2 ml of 1, 2.5, 5.0 and 10 M HCl, giving final concentrations of about 0.1, 0.25, 0.50, and 1.0 M HCl. The final pH of the urine samples was found ranging from 3.5 to 6.0. The samples were kept in the dark and stored at room temperature for 24 h and 48 h. The samples were extracted as described previously except that 2 ml samples were used instead of 5 ml. During analysis, it was found that these samples contained an interfering peak which co-eluted with the internal standard DHBA peak leading to inaccurate quantitation of the free CATS. Therefore another internal standard, epinine, was used for these 2 experiments on rat urine stability. The use of epinine has been shown to have similar analytical performance as the DHBA (Wong 1992). HPLC analysis was as previously described except that the sample

cycle time was adjusted to 18 min instead of 16 min because epinine eluted 1 min after the DA peak.

The results are summarised in Table 3-10. Two-way ANOVA showed that NA was significantly affected by acid factor ($p<0.001$), but not by duration of storage ($p=0.774$) and there was no interaction of the above 2 factors ($p=0.057$). Further analysis showed that samples stored in 0.50 M and 1.0 M HCl were significantly different from the "0-h" samples. In 1 M HCl sample, urinary free NA increased by 6.8 % at 24 h and by 11.4% at 48 h. This shows that rat urinary free NA increased with increasing acid concentration, probably due to the hydrolysis of conjugated NA.

Free A was also significantly affected by acid concentration ($p<0.0001$), but not by storage ($p=0.819$) and there was significant interaction between these 2 factors ($p=0.003$). However, the pattern was quite different from that of NA. The urine A concentrations was significantly lower at 24 h when compared with the "0-h" samples. These results indicated that the lower acid concentration might not protect A from oxidative destruction. On the other hand, urinary free A concentrations were higher after 48-h. The hydrolysis of conjugated A could explain this. Thus, samples containing 1 M HCl were not significantly different from the 0-h samples after 48 h of storage. Results that were significantly different from the 0-h samples were identified by the Scheffe test and are summarized on Table 3-10.

ANOVA analyses showed that DA was affected by the acid factor ($p<0.001$), but not by duration of storage ($p=0.983$) and there was no significant interaction between these 2 factors ($p=0.743$). All samples stored in acid had significantly lower DA concentration compared to the "0-h" samples. DA concentration decreased with increasing acid concentration. The presence of acid enhanced destruction of DA instead of protecting it. Furthermore, the rate of hydrolysis in high acid concentration was slower than the rate of destruction. The interplay of the 2 processes managed to limit the magnitude of decrease to less than 10%.

Table 3-10: Effects of storing rat urine samples at room temperature over 48 h in different acid concentrations on free CATS.

Storage Conditions	Concentration (nM) Mean ± S.D.			Relative % Change w.r.t. 0-h sample		
	NA	A	DA	NA	A	DA
"0-h" Samples	340±51.5	130±22.5	1069±259.5	/	/	/
0.10 M HCl for 24 h	345±49.6	120±20.7**	1042±243.1	+ 1.5	- 8.2	- 2.6
0.10 M HCl for 48 h	348±46.8	117±22.2@	1038±248.4	+ 2.2	- 9.9	- 2.9
0.25 M HCl for 24 h	345±45.7	119±20.4**	1034±247.8**	+ 1.4	- 8.4	- 3.3
0.25 M HCl for 48 h	350±45.8	119±22.5@	1035±246.4*	+ 3.0	- 9.0	- 3.2
0.50 M HCl for 24 h	352±48.6	119±23.9@	1023±240.9@	+ 3.6	- 8.5	- 4.3
0.50 M HCl for 48 h	353±51.5*	124±22.5*	1025±247.1**	+ 4.0	- 5.1	- 4.2
1.0 M HCl for 24 h	363±53.6@	122±23.0**	979±227.2@	+ 6.8	- 6.5	- 8.4
1.0 M HCl for 48 h	379±51.3@	130±22.2	989±222.3@	+ 11.4	- 0.5	- 7.5

The changes of mean concentrations were compared with the "0-h" samples using Scheffe tests (* for $p<0.05$; ** for $p<0.005$; @ for $p<0.0001$).

Effects of storage duration on acidified rat urinary free CATS at -20 ℃

Twelve rat urine samples were collected as described in the above section, except that 10 ml of 0.5 M HCl were added to each urine container. Each sample was separated into five 2-ml aliquots. The first aliquot was processed immediately as described above. Other aliquots were frozen and stored at -20 °C for 1, 2, 3 and 4 weeks. At the end of the storage duration, an aliquot was thawed and analysed.

Storage had no significant effect on rat urinary free NA ($p=0.326$), but it was significant for A ($p<0.0001$) and DA ($p=0.0016$). The results are summarized in Table 3-11. Urinary free A concentrations in all frozen samples were significantly lower. The magnitude of decrease ranged from 20% to 35%. These results showed that the amount of acid preservative used was not sufficient to protect from oxidative destruction. After storage for one week, DA decreased significantly compared to the "0-h" samples ($p<0.05$). On the other hand, storage for 4 weeks had no significant effect compared to "0-h" samples. The rat urinary free DA again decreased in the presence of acid, but gradually increased with longer duration period. The use of 0.5 M HCl and storage at -20 °C was acceptable for preserving rat urinary free NA and DA since the variation of concentration was only about 5%. However, this method is not suitable for measuring urinary free A.

Table 3-11: Effects of storing acidified rat urine in the dark at -20 °C on free CATS

Time (wk)	Concentration (nM) Mean ± S.D.			Relative % Change w.r.t. 0-h sample		
	NA	A	DA	NA	A	DA
"0-h"	230±37.1	80±19.9	1345±237.3	/	/	/
1	221±43.0	64±26.7*	1275±282.9*	- 3.9	- 20.0	-5.2
2	222±40.7	62±39.9*	1294±315.5	- 3.5	- 22.5	-3.8
3	223±39.4	52±21.1@	1289±319.3	- 3.0	- 35.0	- 4.2
4	224±36.8	56±19.0**	1351±322.9	- 2.6	-30.0	+ 0.4

The changes of mean concentrations were compared with the "0-h" samples using Scheffe tests (* for $p<0.05$; ** for $p<0.005$; @ for $p<0.0001$).

DISCUSSION

A reversed phase ion-pair HPLC method using ECD as detector was developed. The acetate-citric buffer mobile phase was modified from a published method (Weicker *et al* 1984). One reason to adapt this published method was that the mobile phase is marketed commercially by a chromatography company. Similar to most published HPLC methods, modification is required when different columns or particle size of the same packing material is used.

The mobile phase was modified mainly by increasing the % methanol and flow rate (Table 3-3). The objective was to maintain a total elution time of 15 min for all the CATS with baseline separation. Another modification was to replace the 1 mM di-n-butylamine with 2 mM triethylamine because the butylamine was not available in the laboratory. The purpose of amine additive was to saturate the "active sites" of reversed phase column and to improve the between-column precision performance. However, the development of "end-cap" technology has removed the active sites and rendered the use of amines unnecessary. Other experiments (not reported here) using the present system showed that there was no difference in the separation of all 4 analytes with or without the addition of amines in the mobile phase.

For the preparation of the mobile phase, it was necessary to keep the freshly prepared solution overnight before filtering with 0.45 μm aqueous filters. Freshly prepared mobile phase was found to block up the filters very rapidly suggesting that precipitates were present. The ion-pair reagent, octanesulfonate, might take more time to dissolve. After the mobile phase had been used, it could be recycled after filtering. Recycling of the mobile phase provided a more economical system. The number of useful cycles depended on the number of samples analyzed. Since the ECD used in the present system was an amperometric type, only 10% of the electroactive species was involved in the electrochemical reaction (Nyyssönen & Parviainen 1989). The remaining electroactive species were left in the mobile phase causing an increase in the background current for the subsequent cycle. A new mobile phase solution was used when the background current was above 10 nA. Use of a guard column reduced

the damage to the column due to the presence of precipitates in the sample. When the system was idle, the flow of mobile phase was maintained at 0.1 ml/min. Regular washing of the column with different amounts of methanol was done once every 3 months. The same column was used for over 2 years with more than 9000 injections.

Baseline separation of the CATS and the internal standard was satisfactory. Precision of the retention times with freshly prepared mobile phase over 72 h showed a CV of less than 1%. After the mobile phase had been recycled over a period of more than 2 weeks, the retention times began to increase, probably due to the evaporation of methanol from the mobile phase. Since standard solutions and quality control samples were incorporated into every batch of HPLC analyses, small deviation of the retention times was acceptable for identification of the peaks.

The applied potential for amperometric ECD was reported to be around +0.70 V (Oka *et al* 1982, Wu & Gornet 1985, Fosti *et al* 1987, Anderson *et al* 1988). Hydrodynamic votammograms of CATS and the internal standard showed that a lower potential of +0.60 V could be used for quantitation (Figure 3-3). The use of lower potential is beneficial because fewer electroactive species are involved in the electrochemical reaction giving a lower noise baseline. This is especially important when the mobile phase is recycled. Background current for a freshly prepared mobile phase was 0.3 - 0.5 nA. This was lower than that of another HPLC-ECD system of 1.2 - 5.0 nA using an applied potential of +0.65 V (Foti *et al* 1987).

Both the linearity and detection limit of the ECD in the present system was sufficient for the measurement of urinary CATS. With an injection volume of 15 µl, the linearity range of the ECD was from 30 nM to 5000 nM for all the CATS (Figures 3-4). Thus, the lowest detection limit of CATS was 0.45 pmole. By increasing or decreasing the injection volume, the range of measurable concentrations can be expanded. Sensitivity of the detector was not assessed by direct injection of the standard solutions into electrochemical cell because the presence of other components in the mobile phase could affect the electrochemical reactions. For example, the presence of EDTA would enhance the signal to noise ratio as some interfering cations

can be chelated (Nyyssönen & Parviainen 1989). It would be more important to determine the linearity range and detection limit of the complete measurement system after urine samples had undergone preliminary purification procedures.

For the extraction of urinary CATS, a published method using one step alumina was adapted (Davidson & Fitzpatrick 1985). Tedious procedure for the activation of alumina was followed to ensure good precision in recovery of CATS for the extraction step. The recovery performance of alumina varied from lot to lot and between suppliers (Anton & Sayre 1962). The activation procedure reduced the problem.

Urinary CATS were adsorbed by alumina at an optimal pH of 8.6. It was reported that plasma CATS had maximum binding with alumina between pH 8.2 and 8.9 (Bouloux *et al* 1985). In the present method, the optimal pH was maintained by a 3 M Tris buffer solution that was higher than the commonly used 1 M Tris buffer (Foti *et al* 1987). This high buffer concentration was to overcome the interference of high creatinine concentration in some urine samples (Davidson & Fitzpatrick 1985). Furthermore, a freshly prepared reducing solution, containing 2.8 mM of ascorbic acid and sodium metabisulphite, was incorporated into the extraction mixture to protect the CATS from oxidative destruction at alkaline pH. It was reported that A was not affected at pH 8.6 for 5 h (Davidson & Fitzpatrick 1985).

After the CATS adsorbed onto the alumina in the 3M Tris buffer, alumina was washed with 0.1 M Tris buffer, also at pH 8.6, instead of the published 0.1 M barbitone buffer. Preliminary experiment showed that there was no difference in recovery between the use of the 2 buffers. With the use of a diluted buffer for washing, the optimal pH was maintained until the elution step. This was reflected by a higher recovery of over 95% compared to about 70% for those using two to three washes with water (Foti *et al* 1987, Anderson *et al* 1988).

Elution of the CATS from alumina was achieved using 0.1 M HCl solution. Two other acid solutions were also tested (Table 3-4). With all the 3 solutions the

recovery was over 95%. The stronger 0.5 M HCl was not preferred as the high acid concentration could affect the stability of the stationary phase. Furthermore, strong acid concentration could enhance oxidative denaturion of the eluted CATS. The 0.1 M HCl solution had higher recovery than the 0.5 M acetic acid and also it was reported that eluted CATS were unstable in acetic acid solution with sodium metabisulphite as antioxidant (Hugh *et al* 1987).

Although the presence of ascorbic acid protected the CATS during alumina extraction, it also presented a problem in the subsequent chromatographic-ECD analysis. It was found that 42.3% of ascorbic acid in the extraction mixture also adsorbed onto the alumina and 3.3% of it was eluted with the CATS (Hugh *et al* 1987). Adsorption of ascorbic acid may be related to the two adjacent hydroxyl groups on the ring structure of the acid. It is a free radical scavenger and may convert CATS quinone back to the parent compounds by inhibiting the irreversible cyclization to an aminochrome. However, ascorbic acid in the eluant was also oxidized in the ECD and eluted before the CATS during the HPLC separation. Total elution time of the ascorbic acid peak was concentration dependent. When the concentration was high, the elution time overlapped with that of NA and A. These 2 peaks were eluting on a changing slope, making integration of peak areas unreliable. This problem was overcome by adding an accurate amount of alumina, not more than 100 mg to the extraction mixture. Similar interference has been reported in the literature (Hugh *et al* 1987).

The choice of extraction methods for urinary CATS is controversial. Based on an evaluation of analytical specificity, sensitivity, recovery and turnaround time, Wu & Gornet (1985) recommended the use of weak cation-exchange resins followed by alumina extraction. In their report, the lack of specificity was a main problem for the alumina extraction because of many other interfering peaks that appeared on the chromatogram. On the other hand, there have been reports claiming that the use of one step alumina extraction was sufficient for both urine and plasma samples (Foti *et al* 1987, Anderson *et al* 1988). In the present method, ethyl acetate was used to wash

the acid eluant and this improved the specificity of the alumina method by removing interfering electroactive species.

Precision performance of the present method was comparable with other reported methods in the literature. For NA and DA, the intra-batch CV was better than 5% and the inter-batch CV was better than 10% (Tables 3-5 and 3-6). For A, both intra and inter-batch precision performance had CV values about 20%. The characteristic poor precision performance for A was a common observation due to the low concentration of the analyte in the sample. Most reports had intra-batch precision performance of 5 - 6% and inter-batch precision of 10-20% (Moyer *et al* 1979, Moerman & de Schaepdryver 1984, Wu *et al* 1985, Davidson & Fitzpatrick 1985, Parker *et al* 1986, Foti *et al* 1987, Anderson *et al* 1988). Peaston (1988) reported a day-to-day CV of better than 6% using a similar system. However, the 3 levels of urine samples studied had free A concentration ranging from 80 to 480 nM.

Recovery performance of the method was mainly affected by the extraction procedure before the HPLC separation. The recovery for a one step alumina extraction has been reported to be 55-65% (Anderson *et al* 1988), 62-65% (Foti *et al* 1987), at least 75% (Anton & Sayre 1962), 80% (Wu & Gornet 1985) and better than 95% (Davidson & Fitzpatrick 1985). Those extraction methods using water to wash away the buffer before the elution step had lower recovery. The washing of alumina with diluted buffer at optimal pH tended to have higher recovery. The present method had recovery of 98-100% for all three CATS (Table 3-5).

The present HPLC-ECD method could measure urinary free CATS up to 10 μM (Figure 3-5). This linearity range was comparable with published methods, for example 10 μM for NA/ A and 25 μM for DA (Peaston 1988), 11.8 μM for NA and 11μM for A (Parker *et al* 1986). However, the injection volume had to be adjusted.

Preanalytical factors are also important for the reliability of any measurement of urinary free CATS. Due to the characteristic catechol ring structure of the CATS, they are sensitive to oxidative agents and are converted to their quinone forms.

Presence of divalent cations catalyzes the oxidative reaction, especially under alkaline pH condition. CATS are not stable in urine samples because of the presence of different cations and urine samples tended to become alkaline over time. CATS are stable at pH between 2 and 5. Therefore, a large variety of acid preservatives have been reported in the literature (Table 3-1). However, the use of acid preservative had not been studied in detail. The matrices of acidified urine samples could be very complicated as 2 different processes might be going on simultaneously in the samples, namely: the oxidative conversion of CATS and the hydrolysis of conjugated CATS to the free forms.

During the study on effects of acid concentration on aqueous CATS standards, it was found that CATS decreased by 1 - 4 % two hours after the standards were prepared in 1 M HCl solution, probably due to oxidative conversion. Free DA was more susceptible than NA and A. The results explained why some research groups incorporated antioxidants even in their standard solutions, for example: sodium metabisulphite (Moyer & Jiang 1978) and EDTA (Kontur *et al* 1984). It was also suggested that standard solutions should be prepared in dilute acids (Nyyssönen & Parviainen 1989). The present finding suggested that the validity of using concentrated acids as urine preservatives should be questioned. Although the final acid concentration in a 24-h urine collection is lower during the first few hours of collection, the acid concentration is high and may cause denaturation of CATS.

To study the process of hydrolysis, a human urine pool was acidified with different amounts of acids and stored at different temperatures. The results showed that at higher acid concentration and higher temperature there was an increase in measurable free DA and NA probably due to the hydrolysis of conjugated CATS (Figure 3-6). There was no consistent pattern of change for A, probably due to the poor precision of method at low A concentrations.

Before the detailed studies of stability of urinary CATS in different acid and storage conditions, effects of storage conditions for CATS in urine were studied first (Table 3-7). Since HPLC analysis is a sequential process, extracts in the eluants have to wait

for the injection into the analytical column. If the extracts are not stable in the autosampler, the subsequent studies will be invalid. The use of 0.1 M HCl for the elution of adsorbed CATS would not enhance oxidative conversion. Furthermore, ascorbic acid added to the extraction mixture was reported to elute with the CATS in an alumina extraction procedure (Hugh *et al* 1987). The CATS extracts were expected to be stable during the period inside the autosampler. However, concentration of NA increased significantly by 3.7%. This phenomenon cannot be explained by the presence of hydrolyzed conjugated NA since there should not be any in the eluants. Evaporation of the sample causing the increase was unlikely. All samples were capped inside the autosampler. The use of peak area ratios with the internal standard for quantitation provided an effective means of showing evaporation effects. Furthermore, if evaporation was the cause of higher NA results, the other 2 CATS should have also been affected. The most likely explanation is a systematic drift during the assay.

During the project, the HPLC system required system shutdown for maintenance and troubleshooting. Extracts had to be stored properly during this period. Thus, the free CATS in the extracts were also studied for their stability when kept in 4 °C over 2 weeks (Table 3-8). The free DA was unaffected during the studied period. However, concentration of A significantly decreased by over 20% after 1 week. In the present chromatographic system, the presence of ascorbic acid could be conveniently observed as a fast eluting peak near the solvent front. The amount of ascorbic acid in the eluants was also observed to decrease. This demonstrated that the antioxidant protection was important for the stability of A in the eluants. Therefore, it is important to complete the HPLC analysis within one week, if measurement of free A is also required. If the extract needs to be stored for more than one week, an ascorbic acid solution should be added. However, the validity of such a practice needs to be proven by an experiment.

To study the effects of duration of storage of acidified human urine, a final acid concentration of 0.025 M HCl was used. This concentration was selected based on the collection of 24-h urine samples into 2-liter containers with 100 ml of 0.5 M HCl.

This preservative was used throughout this project for human urine collection. In previous experiments, 0.5 M HCl solution had been shown not to increase free CATS for a short period. As more urine sample is added during collection, the acid solution would be diluted. The lower acid concentration would not be sufficient to cause hydrolysis of the conjugated CATS.

Urine samples acidified with 0.025 M HCl were kept at 30 °C to simulate collection conditions by outpatients in tropical regions. There were no significant changes in the urinary free CATS over 48 h (Table 3-9). The same samples were also stored frozen at -20 °C to simulate practical situations of storing batches of samples before analysis. Although significant changes of free DA were detected by statistical analyses, the range of changes was within ±5% of the fresh samples. This magnitude of variation was within the inter-batch precision limits. Both NA and A changed significantly by about 10% after 1 month of storage. Therefore, it would be best to store samples frozen at -20 °C for not more than 1 month. Effects of storage at lower temperature, for example, -70 °C, need to be studied.

Separate studies to identify the optimum preservative conditions for rat urine samples are necessary. Composition of rat urine is different from human urine. For example, the bicarbonate concentration was higher in the rat urine sample. Higher acid concentration may therefore be required. However, the majority of rat urinary total CATS are in the conjugated forms (Wang *et al* 1983) and the problem of hydrolysis in the presence of high acid concentration is also of concern.

To study the effects of acid concentration on rat urinary free CATS, urine samples were first collected in the presence of 10 ml 0.1 M HCl. This was necessary as the rat urine samples tended to be alkaline. The "0-h" samples had pH ranging from 3 to 6. The pattern of changes in rat urinary free NA was similar to that of human urine. Increased acid concentration and increased duration of storage at room temperature increased the concentration of NA, suggesting that the rate for hydrolysis of conjugated CATS exceeded the rate of oxidative conversion. However, increased acid concentration did not preserve urinary free A. Samples stored for 24 h had significantly lower concentrations. With prolonged storage, the concentration

increased gradually. Rat urinary free DA was sensitive to increased acid concentration. The duration of storage did not improve the situation. Study on the effect of acid on CATS standards showed that DA was more susceptible to the conversion than the other 2 CATS. The results may mean that the percentage of conjugated DA in the rat urine is lower. Another possible explanation is that the nature of conjugated DA is different in rat urine compared to that of the human type. It has been reported that glucuronide conjugates are predominant in rat and sulphate conjugates predominant in man (Wang *et al* 1983).

When the rat urine samples were preserved with 10 ml of 0.5 M HCl and stored frozen at -20 °C, both urinary free NA and DA decreased by less than 5% even after 4 weeks. However, the urinary free A decreased significantly by more than 20%. This preservative was not suitable for the measurement of urinary A unless the samples were all measured within the first 48 h.

Although acid preservatives are still popular, the results showed that the use of acid solutions to preserve urine samples did not provide satisfactory results. The interplay of the oxidative conversion and hydrolysis of conjugates is complicated. It is difficult to design an optimal condition for different types of urine. Inter-individual variations in the proportion of conjugated CATS could affect the stability results. Alternative preservatives should be studied in detail. The use of an acidic buffer solution might provide the optimal pH condition. The use of antioxidants is likely to delay the oxidative conversion process upon storage. Furthermore, a lower storage temperature could probably extend the storage duration.

The HPLC method developed for the measurement of urinary free DA was up to the "state of the art" performance. Modifications made to the published sample pretreatment methods improved the efficiency of the method and eliminated the chromatographic interference caused by ascorbic acid present in the alumina extracts. The studies on the effects of acids and storage conditions of urine samples provided a better understanding of the limitation by acid preservatives. Thus, the urine samples collected for the subsequent studies were reliable.

CHAPTER 4

CROSS SECTIONAL STUDIES

IN THE HUMAN

I. INTRODUCTION

To understand the roles of DA and ESTI in natriuresis under physiological conditions, cross sectional studies were conducted. The local Chinese population was selected for these studies. They were allowed to continue their usual diet so that the status of the natriuretic factors and sodium homeostasis were all at equilibrium.

The first two studies were conducted among young healthy university students. In the first study, the interrelationships between the Na,K-ATPase inhibitors, DLI, and sodium excretion in 51 medical students were examined. In the second study, the excretion of electrolytes, DLI and DA in 41 female students was studied. These two studies served to confirm previous findings on the interrelationships among the natriuretic factors in the Chinese population.

For the third study, the DA-sodium relationship was investigated among 3 groups of Chinese subjects: normotensives without a family history of hypertension, normotensives with a family history of hypertension, and hypertensives. At the time of the study, there were no published reports on this relationship in the Chinese population.

In the last study, 164 patients with non-insulin-dependent diabetes mellitus (NIDDM) were recruited. It was previously reported that there was decreased urinary DA in microalbuminuric IDDM and NIDDM patients (Murabayashi *et al* 1989, Patrick *et al* 1990). Thus this population showed a reduced ability to mobilize DA under physiological conditions. It was argued that study of such a population is better than pharmacological manipulation of DA production or DA action.

II. RELATIONSHIP OF URINARY SODIUM EXCRETION AND PLASMA ESTI IN MEDICAL STUDENTS

MATERIALS AND METHODS

Fifty-one healthy medical students collected 24-hour urine samples in plain plastic containers. Urine volumes were recorded. A 5-ml aliquot of each sample was stored at 4 °C before being sent for the measurement of urinary sodium, potassium and creatinine. From each student, 5 ml venous blood was also collected into a lithium heparin tube. Plasma samples were stored in screw-capped tubes at -70 °C until analysis for plasma ESTI.

Urinary measurements were performed on the Astra-8 Clinical Chemistry Analyser (Beckman Instruments, Irvin, CA, USA). Urinary sodium and potassium were measured by an indirect ion-selective electrode potentiometry method. The linearity ranges for sodium and potassium were 10-300 mmol/l and 2-300 mmol/l, respectively. The between-batch precision CV for sodium was 1.2% at 100 mmol/l and 2.0% at 35 mmol/l for potassium. Measurement of urinary creatinine was by the kinetic Jaffé reaction. The linearity range was up to 35 mmol/l and the between-batch precision CV was 2.0% at 8.7 mmol/l. Plasma ESTI was measured as the ability to inhibit purified Na,K-ATPase and DLI by radioimmunoassay as described in Chapter 2. Correlation studies were performed using the WinSTAR program as previously described, and the significance level was set to 0.05.

RESULTS

Among the 51 medical students, there were 42 males and 9 females. Their ages ranged from 20 to 25 years. Urinary excretion of creatinine was used to evaluate complete collection of 24-hour urine samples. A creatinine excretion of < 7 mmol was considered an incomplete collection and all 51 samples were included in the study.

Table 4-1 shows a summary of the results for this study. Since there were overwhelmingly more male students than females, the data were not separated according to sex. There was a large variation of sodium excretion, ranging from 35 to 361 mmol/d. All samples showed measurable inhibition on the purified Na,K-ATPase activity. Four samples had DLI results less than 0.10 nmol/l, the lowest detection limit for the method. There were no significant correlations between the plasma ESTI and excretion of sodium and potassium. Excretion of sodium was significantly correlated with the urine volume ($r=0.4615$, $p<0.001$). The 2 assays for measuring plasma ESTI correlated significantly with each other ($r=0.5152$, $p<0.001$). The results showed that in healthy Chinese medical students, there was no correlation between the excretion of sodium and plasma ESTI.

Table 4-1: Urinary excretion of electrolytes and plasma ESTI in 51 healthy medical students

	Mean	S.D.	Range
Urine volume (l)	1.11	0.30	0.40 - 1.90
Sodium excretion (mmol/d)	161	60.4	35 - 361
Potassium excretion (mmol/d)	28	10.4	11 - 56
Na,K-ATPase inhibitor (% Inhibition)	39.5	11.21	21.1 - 73.0
DLI (nmol/l)	0.23	0.072	<0.10 - 0.38

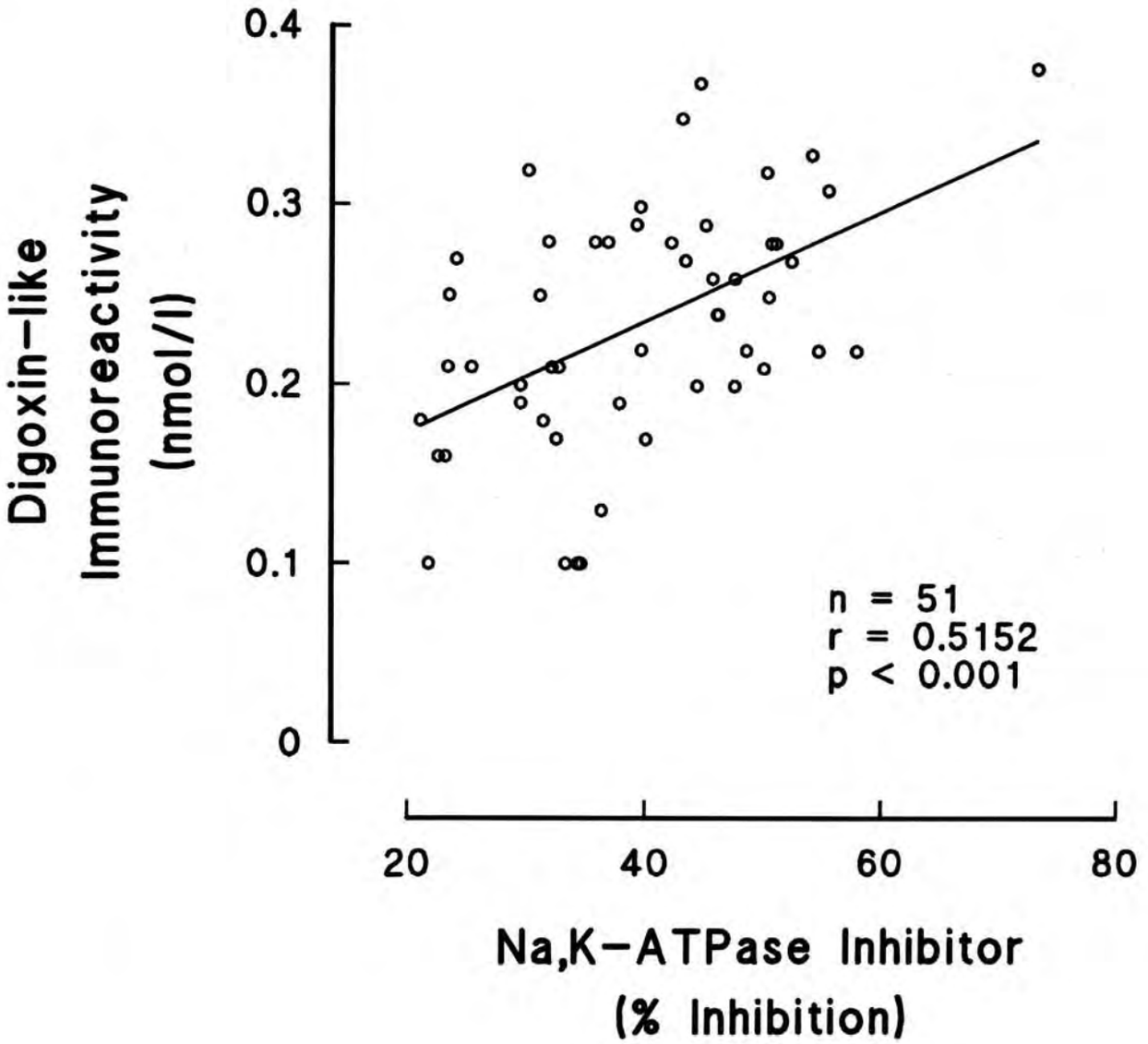


Figure 4-1: **Correlation between plasma Na,K-ATPase inhibition and DLI in 51 Chinese healthy medical students.**

DISCUSSION

The 24-hour urinary sodium excretion in this group of medical students is lower than those reported for Chinese in mainland China (Elliott 1989). The 24-hour urinary excretion of potassium in this group of Hong Kong Chinese is lower than that in many parts of the world, but is similar to that reported from China (Elliott 1989) and Hong Kong (Arumanayagam *et al* 1987).

There is no correlation between the urinary sodium excretion and the concentration of plasma ESTI. A similar finding has been reported (Devynck *et al* 1987). In healthy normal individuals, the kidney can eliminate excess sodium. Thus, the sodium intake does not stimulate the production of ESTI. This finding agrees with the de Wardener's hypothesis (de Wardener & MacGregor 1983). Furthermore, the two methods for measuring ESTI were correlated indicating that the inhibition of purified Na,K-ATPase enzyme and the DLI in healthy subjects are likely to measure the same entity in plasma. However, the correlation between DLI and other methods of measuring ESTI has been reported to be significant in normotensive and hypertensive subjects by some workers (Devynck *et al* 1987, Saitoh *et al* 1988, Graves *et al* 1989) but not by others in hypertensive patients (Hamlyn *et al* 1985). Furthermore, DLI was not correlated with ESTI measured by a quantitative radio-receptor assay in plasma of hypertensive patients (Moreth *et al* 1987). On the other hand, DLI was found to be significantly correlated with inhibition of [^{3}H]-ouabain binding in plasma samples of normal adults, pregnant women, and even neonates (Balzan *et al* 1986). It can be concluded that the correlation between DLI and other methods of measuring ESTI depends on the methodology as well as the origin of the samples.

III. EXCRETION OF URINARY ELECTROLYTES AND NATRIURETIC FACTORS IN YOUNG CHINESE FEMALES

MATERIALS AND METHODS

Forty-one young healthy female Chinese subjects who were taking part in a larger study of determinants of bone mass were asked to collect 24-hour urine samples while they were on their usual intake of nutrients. Urine samples were collected into bottles containing 3 M HCl. The volume of urine samples was measured and aliquots were stored at -20 °C for assay of sodium, potassium, creatinine, calcium, DA and DLI. Measurements of sodium, potassium and creatinine were as described in section I. Urinary excretion of creatinine was used to evaluate complete collection of 24-hour urine samples as previously described. Calcium was measured by a cresolphthalein method on the Parallel Analytical System (American Monitor, Indianappolis, IN, USA). The linearity range was 0.35 - 4.5 mmol/l and between-batch precision CV 1.5% at 2.50 mmol/l. Urinary DA was measured as described in Chapter 3. Urinary DLI was measured as described in Chapter 2, using HEIA.

Correlation studies were performed on the different measured variables. Stepwise multiple regression analysis was also used to further examine the variables with significant correlations. This method searches through all the variables and adds in, at each step, the variable that increases the coefficient of multiple correlation to the greatest extent. The process continues until either the increase in the coefficient is non-significant or all the variables are included.

RESULTS

The age range of the group was 18 - 23 years. Table 4-2 summarizes the mean, standard deviation, and range for the variables measured in this study. Sodium and potassium excretion in this group of students were similar to the medical students in the previous study. All urine samples demonstrated measurable DLI. Table 4-3 shows the correlation between the variables. Sodium excretion was significantly related to the excretion of potassium, calcium, free DA and DLI. Potassium excretion was not related to that of calcium, free DA or DLI. DLI was significantly correlated with calcium excretion. Stepwise multiple regression analysis with free DA as the dependent variable and sodium, potassium, calcium and DLI as independent variables showed that sodium excretion contributed significantly to free DA excretion. Of the variation in free DA excretion, 29.7% was accounted for by variation in sodium excretion. When a similar analysis was done with DLI as the dependent variable, only calcium excretion was significantly related ($r = 0.338$, $p<0.05$) and 11.4% of the variation in DLI was accounted for by calcium excretion.

Table 4-2: Urinary excretion of electrolytes and natriuretic factors in 41 healthy Chinese females.

	Mean	S.D.	Range
Sodium excretion (mmol/d)	132	52.8	54 - 302
Potassium excretion (mmol/d)	39	12.2	18 - 75
Calcium excretion (mmol/d)	2.83	1.21	0.8 - 6.2
Free DA excretion (nmol/d)	1463	304	920 - 2134
DLI excretion (nmol/d)	1.14	0.408	0.42 - 1.98

Table 4-3: Correlation between excretion of sodium, potassium, calcium, free DA and DLI.

	Sodium	Potassium	Calcium	Free DA
Potassium	r = 0.514 (p<0.001)	/	/	/
Calcium	r = 0.348 (p<0.03)	NS	/	/
Free DA	r = 0.545 (p<0.0005)	NS	NS	/
DLI	r = 0.359 (p<0.02)	NS	r = 0.345 (p<0.03)	NS

NS for $p > 0.05$

DISCUSSION

The 24-hour urinary sodium and potassium excretion in this group of young Chinese females is similar to that of the medical students studied earlier (Table 4-1). Mean excretion of calcium in this group was 2.83 mmol/d and lower than reported from Western Countries (Elliott 1989). The 24-hour excretion of calcium in the steady state reflects intestinal absorption of calcium (Nordin 1988) and the amount of calcium absorbed is directly related to the intake of calcium (Nordin *et al* 1988). The low calcium excretion among the Chinese reflects the low dietary intake of calcium (Ho *et al* 1988, MacDonald *et al* 1992).

Excretion of DA and sodium are correlated in normotensive Caucasians (Critchley *et al* 1989) and Japanese (Saito *et al* 1986). The present results confirm the observation for the Hong Kong Chinese. Furthermore, the results show that the major determinant of urinary free DA among the electrolytes is sodium (Table 4-3). Further studies are necessary to explain the relationship between DA and sodium excretion.

Increased dietary salt stimulates the release of ESTI (Doris 1988, Haddy 1990). Using the HEIA, DLI were detected in all urine samples and the excretion of DLI was directly related to the excretion of sodium ($r = 0.359$, $p<0.02$) and to calcium ($r = 0.345$, $p<0.03$). However, stepwise multiple regression analysis showed that only calcium excretion was positively correlated with excretion of DLI. If in normal subjects there is no abnormality in mobilization of DA, sodium intake is unlikely to stimulate the production of ESTI (de Wardener & MacGregor 1983). On the other hand, calcium intake in some manner may stimulate the release of the inhibitor. In the rat, increased calcium intake reduced the plasma concentration of DLI (Doris 1988). However, the relationship between DLI and the calcium excretion in this experiment was a positive one. If low calcium intake is a factor in the pathogenesis of hypertension (Luft & McCarron 1991) then one would expect a negative relationship. Further detailed studies are required to understand this complex interrelationship.

Thus it can be concluded that in young healthy Chinese female subjects, urinary free DA excretion is determined at least partly by sodium intake and the excretion of DLI is independent of sodium intake.

IV. URINARY SODIUM/DOPAMINE RELATIONSHIP IN CHINESE NORMOTENSIVES AND HYPERTENSIVES

MATERIALS AND METHODS

Forty-two healthy Chinese subjects, aged 21-62 years, were recruited randomly from hospital staff, medical students and the community by the Department of Clinical Pharmacology of the Chinese University of Hong Kong. Their family history of hypertension and other individual personal data were recorded, including age, sex, and past medical history. Blood pressure was measured after 5 min sitting by a random zero sphygmomanometer and phase V (disappearance of sound) was taken as the diastolic blood pressure (DBP). The subjects were divided into 2 groups: those without a family history of hypertension (Group A) and those with one or more first-degree relatives suffering from hypertension (Group B). Fourteen hypertensive subjects with normal renal function were also recruited from the Hypertension outpatient clinic at the Prince of Wales Hospital during the same period (Group C). Their medications were stopped for at least 2 weeks before the study.

While the subjects were on their usual diet, each of them was asked to collect one 24-hour urine sample in a plastic bottle containing 25 ml of 5 M HCl solution. During the collection, they were asked to abstain from alcoholic consumption and any medication. The urine volumes were measured and the samples were aliquoted and stored at -20 °C until analysis. Urinary sodium and creatinine were measured as described in section I. Excretion of creatinine was used to evaluate completeness of collection of 24-hour urine samples as previously described. Urinary free DA and NA were measured as described in Chapter 3. Statistical analyses were performed by the WinSTAR program. Correlation studies were done for the relationship between the excretion of sodium and free DA. The inter-group comparison was analyzed by ANOVA, followed by Scheffe's test to test for significant difference between each group. Signficance was set to $p<0.05$.

RESULTS

The 42 normotensive subjects had systolic blood pressures (SBP) below 140 mm Hg and diastolic blood pressures (DBP) below 90 mm Hg. They were further divided into Groups A and B. Group A had 27 subjects with 9 males and 18 females. Group B had 15 subjects with 4 males and 11 females. Among the hypertensive Group C, there were 14 subjects with 4 males and 10 females. Their SBP was above 160 mm Hg and DBP was above 95 mm Hg. Mean arterial pressure (MAP) was calculated according to the formula : MAP = (SBP - DBP)/3 + DBP.

The clinical data and urinary excretion were summarized on Table 4-4. The ANOVA showed that the hypertensives had significantly higher MAP ($p<0.001$). There was no significant difference in the excretion of creatinine, sodium, DA and NA among the 3 groups. Although not statistically significant, the mean sodium, NA and DA excretion were higher in Group C. Furthermore, the MAP of group B is also higher than the Group A. However, the Scheffe test indicated that the difference was not statistically significant.

There was no significant correlation between MAP and the excretion of sodium and DA in any of the groups. A significant positive correlation between the excretion of sodium and DA was observed for Group A ($r = 0.502$, $p<0.01$), while there were no significant correlations for Group B ($r = 0.251$) or Group C ($r = 0.343$) or even a combination of the Groups B and C. Results are also shown on Figure 4-2. A positive significant correlation between excretion of NA and DA was also observed ($r = 0.444$, $p<0.001$).

Table 4-4: **Clinical and urinary excretion data for the study of the relationship between sodium and free DA in Chinese normotensive and hypertensive subjects.**

	Group A	Group B	Group C
N (males, females)	27 (9, 18)	15 (4, 11)	14 (4, 10)
Age (years)	38.1 ± 13.1	41.9 ± 11.2	46.6 ± 9.6
SBP (mm Hg)	104 ± 10.8	110 ± 15	162 ± 16.2*
DBP (mm Hg)	68 ± 7.7	71 ± 7.2	101 ± 8.2*
MAP (mm Hg)	80 ± 8.3	85 ± 9.0	118 ± 10.1*
24-hour urine volume (litre)	1.67 ± 0.51	1.49 ± 0.57	1.73 ± 0.60
Creatinine excretion (mmol/d)	9.1 ± 2.60	8.8 ± 2.52	9.7 ± 2.47
Sodium excretion (mmol/d)	131 ± 43.6	137 ± 29.3	168 ± 77.5
DA excretion (nmol/d)	1495 ± 526.8	1616 ± 433.5	1629 ± 410.3
NA excretion (nmol/d)	183 ± 80.2	184 ± 44.4	192 ± 54.3

Results as mean ± SD
Group A : normotensive subjects without family history of hypertension
Group B : normotensive subjects with family history of hypertension
Group C : hypertensive subjects
* denotes $p<0.001$

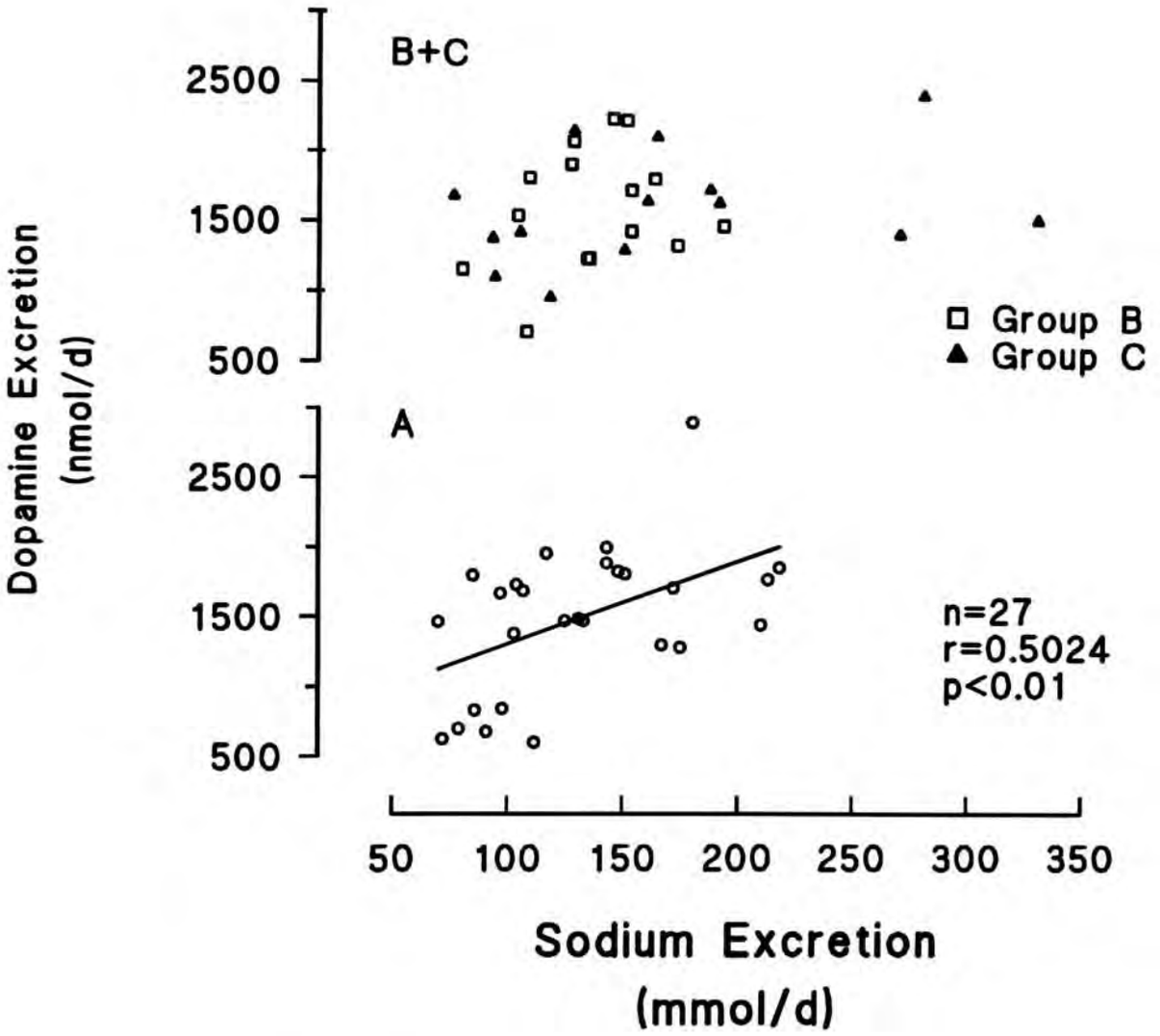


Figure 4-2: Relationship between the excretion of sodium and DA in groups of Chinese subjects.

Group A: 27 normotensives without family history of hypertension
Group B: 15 normotensives with family history of hypertension
Group C: 14 hypertensives

DISCUSSION

Impaired urinary excretion of sodium may be related to the deficiency in mobilization of renal DA in patients suffering from essential hypertension (Lee 1986). In Japan, a strong correlation between urinary DA and sodium was observed in normotensive subjects without a family history of hypertension (Saito *et al* 1986). Family history of hypertension was defined as at least one first-degree relative, for example parents or siblings, having been diagnosed as hypertensive. However, this correlation was not observed in subjects with a family history of hypertension. In another study where the family history of hypertension was not considered, a strong correlation between the excretion of DA and sodium was observed in Caucasians, Zimbabweans and the Thais (Critchley *et al* 1989). However, the same report also showed a lack of correlation in the excretion of free DA and sodium in two other ethnic groups, most of them residing in the United Kingdom. Based on this finding, it was argued that there is a difference among the ethnic groups in conserving salt. The present study was the first study in an ethnic Chinese population. The results showed a strong correlation between urinary DA and sodium in normotensive subjects without a family history of hypertension (Group A). The coefficient of correlation was similar to that reported for Caucasians (Critchley et al 1989). There was no correlation between sodium and DA in normotensives with a family history of hypertension (Group B) or hypertensives (Group C), similar to the Japanese (Saito *et al* 1986).

The loss of the association between sodium and DA excretion in the hypertensive group provides evidence that DA may be an important factor facilitating sodium excretion. The loss of this association may lead to volume expansion and subsequently to development of hypertension. However, the failure to mobilize DA in the kidney may be a consequence of the hypertension process rather than the cause of hypertension. The loss of correlation between sodium and DA excretion in normotensives with a family history of hypertension, suggests that the abnomality of renal DA production in the hypertensive patient is inherited, not acquired (Lee 1993). Furthermore, other studies have also suggested that renal DA receptors are up-regulated in hypertensives showing that the production of renal DA is defective and

insufficient (Lee 1993). On the other hand, studies in the spontaneously hypertensive rat model showed that there is a defect in the DA_1 receptor G protein coupling to adenylate cyclase in the proximal convoluted tubule of the kidney (Kinoshita *et al* 1989).

No statistically significant differences in the excretion of DA were found among the 3 groups of subjects but there was a trend for DA excretion to increase from Group A to C (Table 4-4). This trend is in contrast to reports showing DA excretion to be lower in hypertensives than in normotensives (Lee 1993). Furthermore, the urinary DA excretion was significantly lower in young normotensives with a family history of hypertension compared to age-matched normotensives without a family history. On the other hand, there are also reports showing that free DA is higher in borderline essential hypertensives (Kuchel 1989) and in young patients with essential hypertension (Saito *et al* 1994). To further complicate the issue, it has also been reported that there is no difference on DA excretion between hypertensive patients and age- and sex-matched healthy volunteers (Romero *et al* 1992).

Discrepancies between the results of these studies could be due to the differences in the study populations. A study of 1000 subjects using a specific HPLC method showed that there were age- and sex-related differences in the excretion of DA in adults aged 17-88 years (Gerlo *et al* 1991). Urinary 24-hour DA excretion was significantly higher in the males than the females for all the age groups studied. Furthermore, an inverse relationship between age and DA excretion was also observed for both sexes. The decrease in DA excretion was about 6.5% per decade. It was postulated that the age-dependent decrease may be related to a decrease in the amount of L-dopa or a diminished activity of dopa decarboxylase in the kidney. Although the cause is unknown, it may play a pathophysiological role in the reduced ability of the elderly to handle a sodium load. However, when the results were expressed in relation to the excretion of creatinine, the excretion of DA was unrelated to age in men but still significant in women. This is probably due to the parallel decrease in creatinine excretion with age (Morgan *et al* 1978). On the other hand,

urinary creatinine excretion is affected by diet and exercise, thus excretion of CATS should not be expressed in relation to creatinine (Jenner *et al* 1987).

It would be very interesting to study the DA and sodium relationship in different age groups. Reported sodium and DA relationships were observed mostly in the younger population. Since blood pressure increases with age, the age-dependent decrease in DA could be one contributing factor. In this study, there was no significant difference in age among the 3 groups, thus, the age-dependent effect of DA excretion is not a factor. However, distributions of men and women in the 3 study groups were uneven. To understand the sodium and DA relationships, more subjects would need to be recruited in the different groups.

There was a positive correlation between the excretion of DA and NA. This correlation has also been reported in a group of young hypertensives (Saito *et al* 1994). Urine NA excretion reflects sympathetic nervous activity. This correlation suggests that renal DA production is in part under the influence of sympathetic nervous activity. It is also possible that renal DA stimulates sympathetic nervous discharge via dopaminergic receptors, or renal NA is a link between sodium intake and tubular DA production. However, such a correlation is not compatible with the theory that DA inhibits NA release from sympathetic nerves via DA_2 receptors (Goldberg 1984). Another possible explanation is a pre-analytical factor. Acid hydrolysis of conjugated NA and DA in the 24-hour urine collection might give falsely high NA and DA as described in Chapter 3. More studies are required to understand this relationship.

V. URINARY DA EXCRETION AND PLASMA ESTI IN NORMOTENSIVE AND HYPERTENSIVE NON-INSULIN DEPENDENT DIABETES MELLITUS (NIDDM) PATIENTS

MATERIALS AND METHODS

This study was a collaboration between the Departments of Chemical Pathology, Clinical Pharmacology and Medicine at the Prince of Wales Hospital, The Chinese University of Hong Kong. Measurement of plasma atrial natriuretic peptide (ANP) was performed by the Department of Medicine. Measurement of other plasma and urinary analytes was performed by the Department of Chemical Pathology.

A total of 164 Chinese patients with NIDDM, 67 males and 97 females, aged from 25 to 75 years, were recruited from the Diabetes Clinic at the Hospital and were studied on 2 occasions over a 6-week period. All patients received their usual dietary and oral hypoglycaemic therapy and none was receiving insulin. Anti-hypertensive treatment was taken by 102 patients, but was withdrawn for at least 2 weeks before the study.

On each of the two study visits, supine (after 5 min of rest) and erect (after 2 min of standing) BP was measured by a single research nurse using a random zero sphygmomanometer. Phase V (disappearance of sound) was taken as the DBP. MAP was calculated as described in section III. The mean of the supine and erect MAP is shown for each subject unless otherwise stated. After the BP measurement, venous blood was taken for the measurement of plasma electrolytes, urea, creatinine, hemoglobin A_1 (HbA_1) , ANP, renin, aldosterone and ESTI. Twenty-four hour urines were also collected for the measurement of electrolytes, creatinine, albumin (UAE), and CATS. Urinary tract infection was excluded by examination of a midstream specimen of urine.

Plasma ANP measurement was performed as described (Yandle *et al* 1986). Briefly, blood sample was collected in an EDTA bottle containing Trasylol. Plasma ANP was extracted by C18 Sep Pak cartridges (Millipore, Milford, PA, USA) pre-washed with methanol and 4% acetic acid. After the plasma was loaded onto the cartridge, the resin was washed with 4% acetic acid, and then ANP was eluted with an aqueous solution containing 80% ethanol and 4% acetic acid. The eluant was dried under an air stream and the residue was reconstituted in buffer solution for radioimmunoassay. The plasma extract was incubated with a rabbit antiserum against the c-terminal region of αhuman-ANP for 21 h at 4 °C. Then, [^{125}I] ANP was added to incubate with the reaction mixture for another 24-hour at 4 °C. The bound and free ANP were separated by addition of goat anti-rabbit antiserum. The precipitated bound fraction was counted in a gamma counter. Intra- and inter-assay coefficients of variation were both between 11 and 14% at varying concentrations of ANP in human plasma. The detection limit was 10 pg/ml.

The following analytes were measured by the routine laboratory of the Department of Chemical Pathology at the Prince of Wales Hospital. HbA_1 was measured by agarose gel electrophoresis using a commercial kit (Ciba Corning Diagnostics Corp., Alto, CA, USA). The inter-assay CV was better than 8%. Plasma renin concentration was measured by a radioimmunoassay kit (ERIA Diagnostics Pasteur, France). The inter-assay CV was 11% and the detection limit was 7.5 ng/l. Plasma aldosterone concentration was measured by a coated tube radioimmunoassay kit (Diagnostic Products Corp., CA, USA). The inter-assay CV was 12% and the detection limit was 68 pmol/l. UAE was determined by immunoturbidimetry on the centrifugal analyser Cobas Bio (Cheung & Swaminathan 1989). The inter-assay CV was better than 7%. The detection limit was 2.5 mg/l.

Plasma ESTI was measured by the inhibition of purified Na,K-ATPase activity and DLI by the CEDIA HEIA as described in Chapter 2. Urinary DA and NA concentrations were measured as described in Chapter 3. Plasma and urinary electrolytes and creatinine were measured as described in the previous sections of this Chapter.

Statistical analyses were performed with the WinSTAR statistical package on a personal computer. The mean values of all variables obtained on both visits were used for statistical analyses. Since the distributions of UAE, sodium, DA and NA outputs and plasma ANP, renin, aldosterone and ESTI concentrations were skewed, these data were log transformed for some statistical analyses. Their results were expressed as medians and their ranges. Other results were expressed as mean ± SD. Correlation coefficients were calculated by Pearson's method. Stepwise multiple regression analysis was used to study the interrelationships between these variables. Comparisons between groups of the subjects were performed using ANOVA. A p value of less than 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Grouping of patients according to urine albumin excretion

Patients were divided into 3 groups according to UAE results. Based on the mean value of two measurement of UAE, 86 patients had normoalbuminuria (UAE <30 mg/d), 48 patients had microalbuminuria (UAE between 30 - 300 mg/d) and 30 patients had macroalbuminuria (UAE > 300 mg/d). Table 4-5 summarizes the clinical data and the ANOVA results of these 3 groups. There were no significant difference in age and glycaemic control between these 3 groups of patients. The MAP was higher in patients with abnormal albuminuria. Urinary sodium output was similar in all 3 groups of patients. Plasma ANP concentration increased with increasing proteinuria, whereas urinary DA output was lower in proteinuric patients.

Creatinine clearance and urine NA output showed a similar pattern to that of urine DA output. Plasma ESTI concentrations were not different among the 3 groups of patients. Finally, plasma renin concentration increased whereas plasma aldosterone decreased with increasing albuminuria.

UAE correlated positively with plasma ANP concentration ($r=0.312$, $p<0.0001$) and negatively with urine DA output ($r= -0.179$, $p<0.05$). Supine systolic blood pressure (SSBP) also showed a similar correlation with ANP ($r=0.579$, $p<0.0001$) and DA output ($r= -0.221$, $p<0.01$). These inter-relationships are shown on Figures 4-3 and 4-4. There was also a significant correlation between UAE and SSBP ($r=0.429$, $p<0.0001$).

Table 4-5: Clinical data, plasma concentration and urinary output of analytes for subjects grouped according to albumin output.

	Urine Albumin Output < 30 mg/d	Urine Albumin Output > 30 and <300 mg/d	Urine Albumin Output > 300 mg/d	ANOVA F ratio
Clinical Data				
Males : Females	36 : 50	20 : 28	11 : 19	/
MAP (mmHg)	104.3 ± 15.9	111.3 ± 16.7	112.1 ± 13.0	4.523*
Age (years)	52.5 ± 9.8	56.8 ± 12.2	55.3 ± 12.5	NS
Plasma concentration				
HBA_1 (%)	10.1 ± 1.7	10.6 ± 2.2	10.5 ± 1.7	NS
Sodium (mmol/l)	140.1 ± 2.2	140.4 ± 2.0	140.4 ± 2.3	NS
ANP (pg/ml)	34.3 (9.0-132.3)	38.6 (14.7-135.2)	43.0 (20.2-141.0)	4.485*
Na,K-ATPase Inhibitor (nmol/l)	5.0 (4.9-16.9)	5.6 (4.9-13.5)	5.1 (4.9-15.6)	NS
DLI (µg/l)	0.10 (0.07-0.27)	0.09 (0.07-0.18)	0.11 (0.07-0.20)	NS
Renin (ng/l)	25.4 (7.4-192.0)	23.1 (7.4-271.0)	46.8 (7.4-271.0)	6.312**
Aldosterone (pmol/l)	338 (83-1336)	330 (82-889)	317 (68-590)	3.280*
Daily Urine Output				
Creatinine clearance (ml/min)	82 ± 28.6	78 ± 30.5	63 ± 29.0	4.358*
Sodium (mmol)	154 (33-289)	142 (53-329)	159 (36-230)	NS
Dopamine (nmol)	1370 (150-3312)	1183 (181-2794)	1110 (310-3475)	3.322*
Noradrenaline (nmol)	164 (35-1077)	158 (26-288)	114 (24-271)	4.674*

Results are shown in mean ± SD or median (range).
NS for $p>0.05$; * for $p<0.05$; ** for $p<0.01$; @ for $p<0.001$; @@ for $p<0.0001$

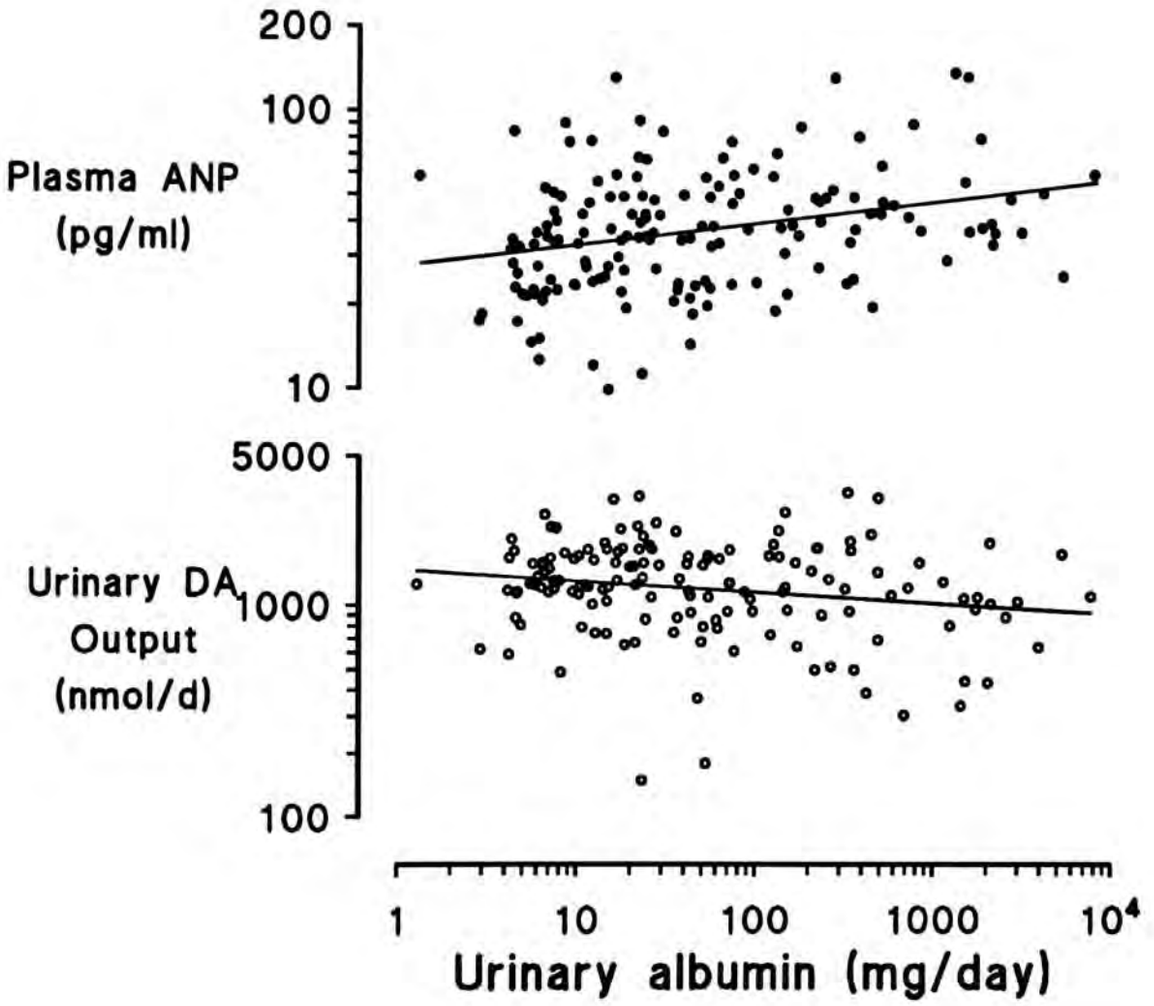


Figure 4-3: Relationships between UAE, urinary DA and plasma ANP in 164 NIDDM patients.
All values of UAE, urinary DA and plasma ANP were plotted on logarithmic scales.

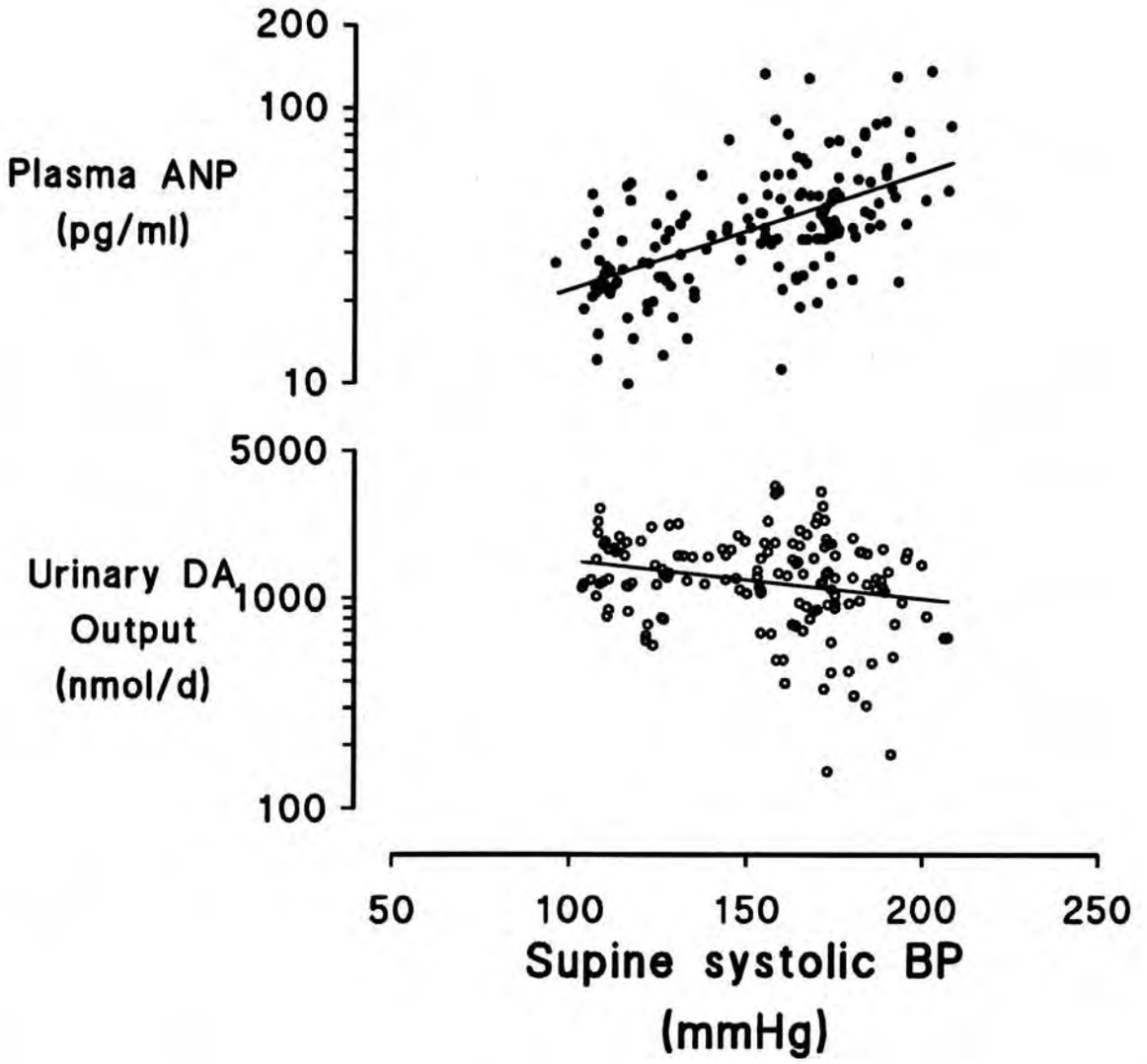


Figure 4-4: Relationships between SSBP, urinary DA and plasma ANP in 164 NIDDM patients.

Values of SSBP was plotted on a linear scale; while both urinary DA and plasma ANP were plotted on logarithmic scales.

Inter-relationships between the measured variables

Since some the variables were inter-correlated, stepwise multiple regression analysis was used to identify the most significant relationship. Tables 4-6 summarizes the standardized regression coefficients (β) and their significant level.

MAP is dependent on a number of factors and it correlates with ANP, Na,K-ATPase inhibitor, sodium excretion and age. Urine excretion of sodium was significantly correlated with the creatinine clearance and DA excretion and weakly correlated with albumin excretion. DA excretion significantly correlated with NA and sodium excretion and weakly with creatinine clearance.

Simple correlation analysis showed that there was a weak inverse relationship between plasma ANP and urine DA output ($r=-0.1626$, $p=0.0492$). The correlation coefficients between plasma ANP and urine DA output were negative in both microalbuminuric ($r=-0.192$, $p=0.2057$) and macroalbuminuric ($r=-0.280$, $p=0.1336$) patients. When these 2 groups were combined and analyzed, the relationship became more significant ($r=-0.236$, $p=0.0417$). With stepwise multiple regression, however, plasma ANP concentration was not significantly correlated with urinary DA output .

Table 4-6: **Inter-relationship between age, mean blood pressure, sodium output, dopamine output, noradrenaline output, albumin output, creatinine clearance, ANP, Na,K-ATPase inhibitor, DLI, renin, aldosterone, using stepwise multiple regression anlysis.**

Dependent Variables	Mean Arterial Pressure	Sodium Output	Dopamine Output
ANOVA for the regression:			
F ratio	19.94	25.76	49.14
p value	<0.0001	<0.0001	<0.0001
Independent Variables:			
Age	$\beta = 0.177^{*}$	NS	NS
MAP	/	NS	NS
Creatinine Clearance	NS	$\beta = 0.377^{@@}$	$\beta = 0.186^{*}$
Sodium Output	$\beta = 0.209^{**}$	/	$\beta = 0.265^{@}$
DA Output	NS	$\beta = 0.315^{@}$	/
NA Output	NS	NS	$\beta = 0.459^{@@}$
UAE	NS	$\beta = 0.211^{**}$	NS
ANP	$\beta = 0.392^{@@}$	NS	NS
Na,K-ATPase inhibitor	$\beta = 0.299^{@@}$	NS	NS
DLI	NS	NS	NS
Renin	NS	NS	NS
Aldosterone	NS	NS	NS

β for standardized regression coefficient
NS for $p>0.05$; * for $p<0.05$; ** for $p<0.01$; @ for $p<0.001$; @@ for $p<0.0001$

Grouping of patients according to blood pressure

Blood pressure is another factor affecting natriuresis. These 164 patients were divided into 2 groups according to their SSBP. The normotensive group was those with SSBP less than 160 mmHg; while the others with SSBP greater than 160 mmHg were considered hypertensives. Table 4-7 summarizes the ANOVA results for the measured plasma and urine parameters. There were 90 normotensives and 74 hypertensives. The hypertensive group was significantly older than the normotensive group. There was no difference in their glycaemic control, plasma DLI, renin and aldosterone concentration, or urine sodium excretion. The plasma ANP, sodium, Na,K-ATPase inhibitor concentrations and urine albumin outputs were significantly higher in the hypertensives. Figure 4-5 shows the relationship between Na,K-ATPase inhibitor and the SSBP. On the other hand, the creatinine clearance, DA and NA excretion were lower in the hypertensives.

The sodium and DA excretion correlated significantly for both the normotensive and hypertensive groups ($r=0.487$, $p<0.0001$ for nomotensive and $r=0.483$, $p<0.0001$ for hypertensive). Figure 4-6 shows the correlations of the 2 parameters slopes of the regression lines were similar.

Table 4-7: Clinical data and laboratory data for the NIDDM subjects grouped according to supine systolic blood pressure.

	Supine SBP < 160 mmHg	Supine SBP ≥ 160 mmHg	ANOVA F values
Clinical data			
Males : Females	44 : 46	23 : 51	/
Age (years)	49.2 ± 10.4	60.5 ± 8.7	56.40@@
Plasma concentration			
HBA_1 (%)	10.5 ± 1.8	10.1 ± 1.9	NS
ANP (pg/ml)	28.5 (9.0-13.7)	47.2(19.1-141.0)	42.46@@
Sodium (mmol/l)	139.5 ± 2.0	141.2 ± 1.9	31.58@@
Na,K-ATPase Inhibitor (nmol/l)	4.9 (4.9-10.6)	7.0 (4.9-16.9)	42.95@@
DLI (µg/l)	0.10 (0.07-0.27)	0.09 (0.07-0.22)	NS
Renin (ng/l)	26.1 (7.4-254)	35.4 (7.4-271)	NS
Aldosterone (pmol/l)	378 (69-1336)	299 (68-982)	NS
Daily Urine Output			
Creatinine clearance (ml/min)	84.5 ± 31.6	68.6 ± 25.1	12.33@
Sodium (mmol)	157 (33-329)	134 (36-264)	NS
Dopamine (nmol)	1350 (508-3475)	1188 (150-3282)	9.650**
Noradrenaline (nmol)	183 (60-1077)	134 (24-453)	17.94@@
Albumin	16.1 (2.3-5193)	71.4 (1.3-7547)	20.75@@

Results are shown in mean ± SD or median (range).
NS for $p>0.05$; * for $p<0.05$; ** for $p<0.01$; @ for $p<0.001$; @@ for $p<0.0001$

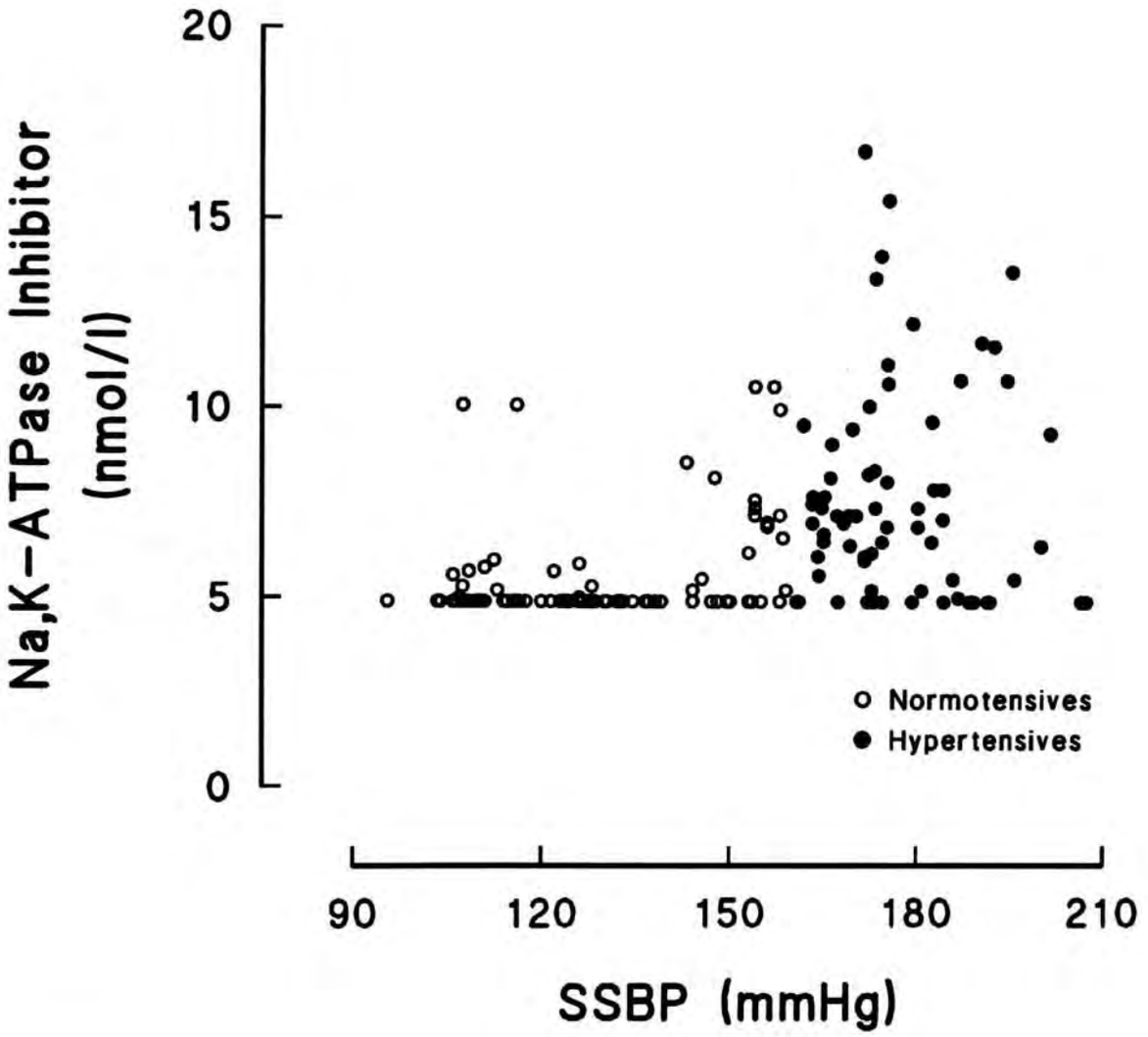


Figure 4-5: **Relationship between plasma Na,K-ATPase inhibitor and SSBP in 164 NIDDM patients.**

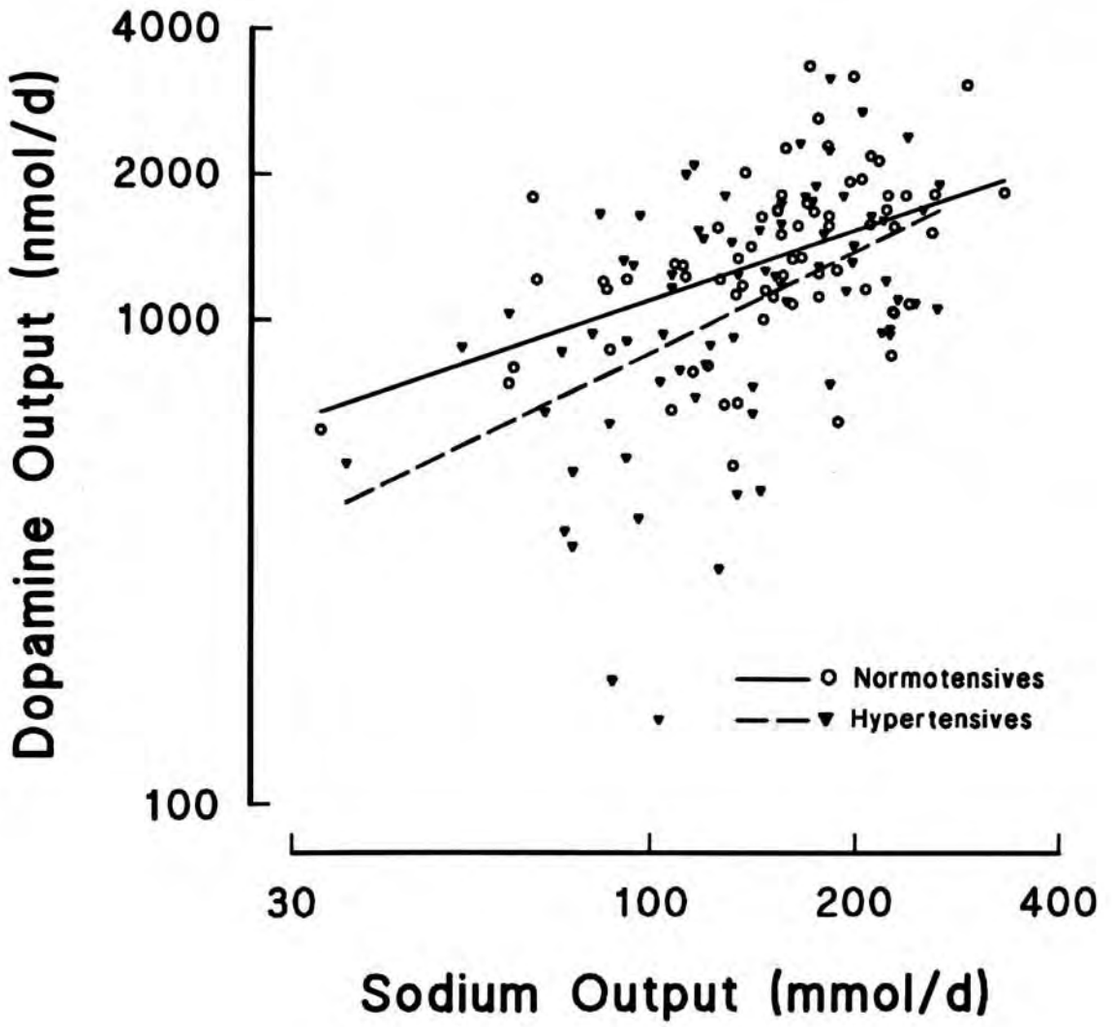


Figure 4-6: Correlation between urinary excretion of sodium and DA in normotensive and hypertensive NIDDM patients.

Urinary DA and sodium values were both plotted on logarithmic scales

DISCUSSION

There is evidence that patients with IDDM and NIDDM have an increase in exchangeable body sodium that correlates with their level of blood pressure (Weidmann & Ferrari 1991). The pathophysiology of this phenomenon is not fully understood. To study the contribution of DA to sodium excretion, inhibitors of the L-dopa decarboxylase or antagonists of DA receptors are often used. However, these inhibitors or antagonists are not present naturally and these pharmacological agents may have other effects and the results obtained with these agents may not be readily applicable to physiological or pathophysiological states. Decreased urinary DA excretion has been reported in microalbuminuric patients with IDDM and NIDDM (Murabayashi *et al* 1989, Patrick *et al* 1990). Thus, diabetic patients can be used as a model where there is reduced DA production. Furthermore, hypertension is another common complication in NIDDM patients. Increased plasma ESTI concentration has been reported in hypertensive patients (Poston *et al* 1981, Hamlyn *et al* 1982). Another natriuretic factor, ANP, has also been reported to increase in hypertensive patients (Sagnella *et al* 1986) and in patients with IDDM and NIDDM (Bell *et al* 1989, Shinoda *et al* 1990, Lieberman *et al* 1991). Intravenous infusion of ANP induces proteinuria in patients with essential hypertension (Suenaga *et al* 1989) and primary glomerular diseases (Zietse & Schalekamp 1988, Ishi *et al* 1989, Hirata *et al* 1991). The population of NIDDM patients appears to be a good model to study the interplay of the various types of natriuretic factors, sodium excretion, proteinuria and effects on blood pressure.

In the present study, 164 NIDDM patients were recruited from the Diabetic Clinic. Among them, 102 were on anti-hypertensive treatment. Effects of anti-hypertensive medication were minimized with a 2-week wash-out period. Each subject had 2 study visits over a period of 6 weeks. They were allowed to continue with their usual diets and hypoglycaemic medication. All measurements and samplings represented an average of the 2 visits. This would provide more representative data for the population.

Decreased DA excretion has been reported in NIDDM patients with microalbuminuria (Murabayashi *et al* 1989). It has been suggested that early microangiopathic changes within the renal tubules may initiate and exacerbate diabetic nephropathy (Pinter & Atkins 1990). Structural damage to the tubule may reduce the amount of L-dopa decarboxylase present and reduce production of intrarenal DA. When the NIDDM patients were divided into 3 groups according to UAE, it was observed that DA excretion decreased significantly with increasing UAE (Table 4-5). Renal function assessed by creatinine clearance also decreased as UAE increased (Table 4-5). These results support the concept that patients with microalbuminuria (Group B) and macroalbuminuria (Group C) suffer from reduced renal function. The observed decrease in DA production is probably due to structural damage to the renal tubules. The differences in DA excretion between the 3 groups cannot be explained by age as there were no significant differences in age among the 3 groups of subjects (Table 4-5).

Despite differences in DA excretion between the three groups, there was no signficant difference in urine sodium excretion (Table 4-5). This suggests that sodium balance is maintained by other natriuretic factors. Increased circulating ANP in NIDDM patients has also been reported (Shinoda *et al* 1990). It is possible that increase in ANP is a compensatory phenomenon and helps to promote sodium excretion. Such a complementary role between plasma ANP and urine DA is further supported by their inverse relationship with UAE (Figure 4-3).

There was a significant increase in MAP with increasing UAE (Table 4-5). Stepwise multiple regression shows that MAP correlated significantly not only with age, sodium excretion and ANP, but also with the Na,K-ATPase inhibitor (Table 4-6). The correlation between Na,K-ATPase inhibitor with MAP has been reported for essential hypertensive patients (Poston *et al* 1981, Hamlyn *et al* 1982). When the NIDDM patients were grouped according to their SSBP, the hypertensive group was older than the normotensive group (Table 4-7). There was no difference in HBA_1 concentration between the 2 groups indicating that there was no difference in glycaemic control. Hypertensive NIDDM patients were associated with higher

proteinuria, plasma sodium, ANP and Na,K-ATPase inhibitor concentrations. Figure 4-5 shows the correlation between plasma Na,K-ATPase inhibitor and SSBP.

Hypertensive NIDDM patients had significantly lower urinary DA outputs (Table 4-7), probably due to damage to the renal tubule as discussed above. This was supported by the presence of increased UAE and decreased creatinine clearance. When the urinary DA and sodium excretion were analyzed in both the normotensive and hypertensive groups, there was significant correlation between them (Figure 4-6). This was in contrast to the results observed in section IV of this Chapter, where the correlation between urinary sodium and DA relationship was lost in the hypertensive as well as normotensive subjects with a family history of hypertension. Subjects in the previous study had normal renal function. The dissociation of the DA-sodium relationship in that group of hypertensives might be due to a genetic defect in the production of intra-renal DA (Lee 1993). The hypertensive NIDDM patients in this study maintained the correlation between sodium and DA excretion. It is unlikely that they have the same genetic defects as described for patients studied in section IV.

Other natriuretic factors were also measured in this study. Urine NA excretion was significantly decreased with increasing UAE (Table 4-5) and increasing blood pressure (Table 4-7). This indicates that there was reduced renal sympathetic nervous activity in patients with increasing UAE and MAP. There was a strong positive correlation ($r > 0.45$, $p < 0.0001$) between urine NA and DA excretion (Table 4-6a). A similar correlation was observed previously in section IV.

The renin-angiotensin system is another system that affects natriuresis. Decreasd plasma renin and aldosterone concentrations facilitate sodium excretion. Plasma aldosterone decreased significantly with increasing UAE (Table 4-5) as expected. Plasma aldosterone was lower in the hypertensive group, but this was not statistically significant (Table 4-7). It was surprising to find that plasma renin concentration increased, as opposed to plasma aldosterone concentration, with increasing UAE (Table 4-5). Furthermore, in the hypertensive NIDDM patients, the plasma renin concentration also was higher although not statistically significant. It has been

suggested that plasma renin behaves paradoxically in patients with renal failure, due to reduced metabolic clearance rate of the enzyme (Rosenberg *et al* 1994). NIDDM patients with increasing UAE and blood pressure had reduced creatinine clearance. Thus the paradoxical increase in renin could be explained by the diminished renal function. Furthermore, plasma renin concentration, not bioactivity, was measured in this study. It is possible that some renin molecules measured is not bioactive. Higher plasma renin may lead to higher concentrations of angiotensin II in the circulation. It has been demonstrated that infusion of angiotensin II in subpressor doses in man can reduce DA excretion (Eadington *et al* 1991).

Based on the results of the present study, it can be postulated that, in patients with NIDDM, early renal tubular damage may result in impaired sodium excretion, in part through defective DA mobilization. A compensatory rise in plasma ANP concentration in response to plasma volume expansion and to hypertension may limit the renal sodium retention, but this could also contribute to worsening of albuminuria and possibly renal damage. This may lead to a further reduction in the urinary DA response to salt intake and initiate a vicious cycle involving sodium retention, hypertension and albuminuria. Diminished renal function also leads to the paradoxical increase in renin which may contribute to further reduction in DA production. The increase in plasma Na,K-ATPase inhibitor observed in these patients may contribute to the development of hypertension.

CHAPTER 5

VOLUME EXPANSION STUDIES IN THE HUMAN

I. INTRODUCTION

Sodium excretion is the major mechanism for the maintenance of ECF volume. There are a large variety of mechanisms to increase sodium excretion in order to maintain homeostasis. To study the role of various factors influencing sodium excretion, the ECF volume is altered experimentally and the effects studied. To expand ECF volume three common methods are used: headout water immersion, acute saline infusion and oral salt loading. This Chapter reports the effect of expanding the ECF volume by these three different methods on DA and ESTI in order to examine their role in sodium excretion.

Section II describes the effect of headout water immersion in 7 Caucasian females. This is the only experiment studied in non-Chinese subjects in the thesis. Headout water immersion is a method to induce prompt redistribution of circulating blood volume by the pressure gradient effects on the cardiopulmonary receptors. When a subject is allowed to sit in a water tank with the body immersed in water up to the neck at thermal neutrality, the central blood volume, intracardiac volume, central venous pressure and cardiac output are all increased. The increase in central blood volume produces natriuresis and diuresis, without changes in plasma osmolality and serum electrolyte concentrations. The detailed mechanism and renal effects of headout water immersion has been recently reviewed (Epstein 1992). An advantage of this method is that endogenous plasma constituents are only affected by the process itself, as there is no alteration in dietary intake or infusion into the blood stream. There have been conflicting reports on the role of DA in sodium excretion induced by this method. Furthermore, very few studies have examined the role of ESTI.

Section III describes the experiment to study the effect of volume expansion by intravenous saline infusion. ECF volume can be expanded acutely by this method to elicit prompt natriuresis. However, it is invasive and involves venous catherization of the subjects. There is no standardized protocol for the volume and duration of the saline infusion. Plasma concentrations of the different components are diluted by the

infused saline. Biological actions of some analytes can be affected, especially those depending on the plasma protein concentration. Like in headout water immersion, there have been conflicting reports on the role of DA, and very few reports on the role of ESTI in the sodium excretion induced by saline infusion.

The last 2 sections in this Chapter describes the effect of dietary salt loading in healthy Chinese subjects. This method simulates what happens in our daily life. According to the hypothesis of the pathogenesis of essential hypertension, failure to excrete sodium promptly in response to increased dietary salt intake initiates the pathological process (de Wardener & Clarkson 1985). This method can be used with a large number of subjects without using expensive equipment. However, to maintain the subjects on a constant dietary sodium intake can be difficult as most processed foods contain high amounts of sodium. The mechanism of inducing natriuresis is different from that of intravenous infusion as it has been shown that the liver plays a significant role in maintaining sodium homeostasis during oral salt loading (Morita *et al* 1993). In these studies, the roles of DA and ESTI in sodium excretion were examined with subjects on their usual diets and on a controlled diet.

II. VOLUME EXPANSION BY HEADOUT WATER IMMERSION

MATERIALS AND METHODS

Serum and urine samples were collected from a study which was designed to investigate the effect of the menstrual cycle on sodium handling (Bisson *et al* 1992). Ten healthy Caucasian female volunteers aged 18 - 30 years (median 24.5) with regular menstrual cycles participated in a headout immersion study. The women were not taking any medication and none of them complained of symptoms suggesting premenstrual syndrome. All subjects gave informed consent and the study was approved by the district ethics committee in the United Kingdom.

Subjects were studied on their normal diets. Each was asked to keep a record of her dietary intake on the three days preceding the first study and followed a similar diet before the second study to reduce within-subject variability in sodium excretion. This was then assessed by a 24-h urine collection immediately preceding each study.

Each subject was immersed twice: once between day 7 - 10 of the follicular phase and once between day 18 - 24 of the luteal phase of the same cycle. After an overnight fast, the subjects took a 400-ml water load an hour before the start of the study. They continued to take 200 ml of water hourly during the study to maintain hydration. For the first hour, they sat outside the tank (preimmersion control hour), followed by 3 h of immersion and then a final postimmersion hour outside the tank to allow all the changes to return to normal. The subjects emptied their bladder at the end of each hour by leaving the tank briefly during the immersion hours. Blood samples were taken through an indwelling cannula. They were given lunch during the final hour.

At each period, 25 ml of venous blood was taken and divided into chilled tubes, kept on ice, centrifuged at 4 °C for 10 min, and serum separated and frozen within 30 min. The serum samples were stored at -20 °C. Portions of the urine samples were acidified for the measurement of urinary free CATS and frozen at -20 °C. Aliquots of

the frozen serum and urine samples were sent to Hong Kong for the measurement of urinary free CATS and plasma ESTI.

Urine sodium, potassium, calcium and creatinine; serum sodium, potassium, creatinine, calcium, and progesterone; plasma renin activity, aldosterone and ANP were measured in the United Kingdom as described (Bisson *et al* 1992). Results of the urine samples and plasma electrolytes were sent with the samples to Hong Kong. Plasma ESTI was measured by the inhibition of purified Na,K-ATPase activity and DLI using the homogeneous enzyme immunoassay as described in Chapter 2. Urinary free DA and NA were measured as described in Chapter 3.

Results of urine and plasma samples were expressed in mean ± standard error of the mean (SEM). The pre-immersion control data were compared between the two cycles using a paired t-test for normally distributed data or a Wilcoxon signed rank test when the data were not normally distributed. If there was no difference in control values, the responses to immersion were compared using ANOVA for repeated measurement. Data were log transformed when necessary to normalize the distribution. When there was a difference in control values, the data were converted to relative changes to allow comparisons of the response to immersion to make between the two phases of the cycle using the ANOVA test.

RESULTS

Seven of the 10 women ovulated as assessed by a 6-fold increase in the mid-luteal phase serum progesterone level (Table 5-1). Only samples from these 7 subjects were analyzed. There was no renal sodium and water retention during the luteal phase of the menstrual cycle (Bisson *et al* 1992). There were no differences in body weight, creatinine clearance, basal sodium excretion and plasma ANP between the 2 phases of the cycle. There was a significant rise in basal progesterone, aldosterone and renin activity in the luteal phase. Renal and hormonal responses to headout immersion including sodium and calcium excretion, elevation of ANP, suppressions of aldosterone and renin activity are identical in the 2 phases of the cycle. Some relevant data are tabulated on Table 5-1.

Only 3 of the 7 subjects had measurable DLI in most of their samples. Similarly, most urine samples had urinary free A lower than the lowest detection limit. Thus, these 2 parameters were not analyzed.

Using the Wilcoxon signed rank test, there was no significant difference between the two phases of menstrual cycle in the pre-immersion control values for plasma ESTI, urinary free NA and DA. Examining the distribution of data, plasma ESTI and urinary free NA were log transformed for the ANOVA test. There were no significant differences for the pre-immersion control results between the 2 phases. To reduce the intra-individual variation, results of the 2 phases were averaged for further analyses. Table 5-2 shows the changes for NA, DA excretion and plasma ESTI. Figure 5-1 shows the relative % changes of these parameters and sodium excretion. There was no significant change in DA excretion during the 5 h. There was a significant change in NA excretion during the 5 h ($F=48.68$, $p<0.0001$). Free NA excretion was suppressed throughout the 3 h of immersion by more than 50%, and it increased by 180% during the post-immersion recovery period. The ANOVA test also showed significant changes in plasma ESTI ($F=7.534$, $p=0.0007$). The plasma concentration increased by about 10% and peaked at the last hour of immersion. During the recovery period, the plasma ESTI concentration decreased by 50% compared to the control period.

Table 5-1: Relevant control data of the follicular and luteal phases during a menstrual cycle of 7 ovulating women (Bisson *et al* 1992).

	Follicular Phase	Luteal Phase	Significanc
Weight (Kg)	51.9 ± 1.5	51.8 ± 1.5	NS
24 h urine volume (l)	1.0 ± 0.08	1.2 ± 0.15	NS
24 h urine sodium (μmol/min)	69 ± 4	73 ± 7	NS
24 h creatinine clearance (ml/min)	129 ± 19	118 ± 8	NS
24 h urine potassium (μmol/min)	40 ± 3	43 ± 4	NS
ANP (pg/ml)	25 ± 5	21 ± 2	NS
Serum progesterone (nM)	1.3 ± 0.4	34.4 ± 2.4	p = 0.002
Plasma aldosterone (pM)	310 ± 24	1077 ± 202	p = 0.01
Plasma renin activity (ng/ml/h)	1.01 ± 0.1	1.97 ± 0.4	p = 0.02

Table 5-2: Excretion of NA and DA and plasma ESTI concentration in 7 ovulating women during and after the headout immersion study.

The results are shown as mean ± SEM and obtained from the average of results between the follicular and luteal phases.

Time	NA excretion (pmol/min)	DA excretion (pmol/min)	Plasma ESTI (nM OE)
Pre-immersion	94 ± 9	1125 ± 54	6.61 ± 1.31
Immersion 1 h	44 ± 4	1186 ± 105	8.57 ± 1.31
2 h	39 ± 5	1093 ± 51	9.01 ± 1.51
3 h	54 ± 6	1079 ± 62	7.53 ± 1.17
Post-immersion	171 ± 17	936 ± 100	4.69 ± 0.92

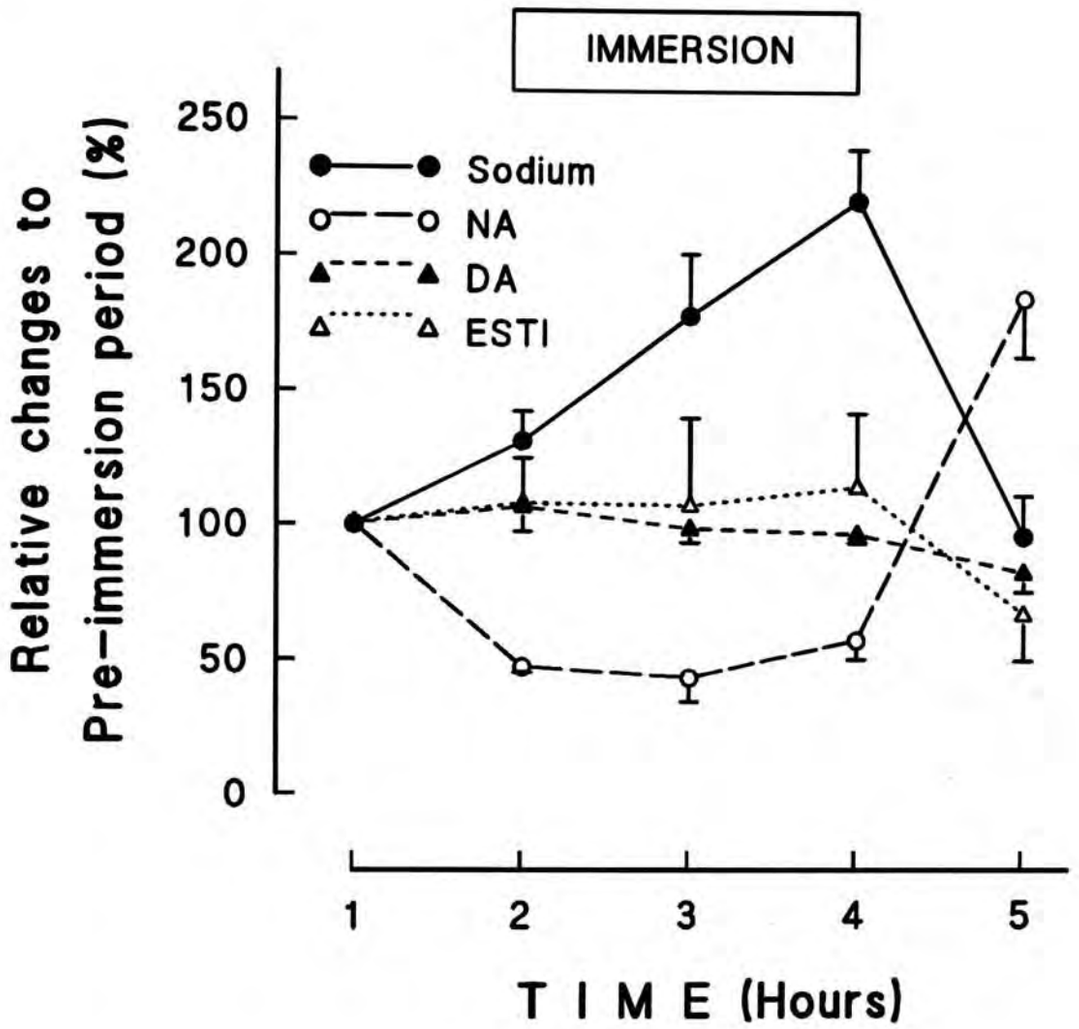


Figure 5-1: Relative changes in the excretion of sodium, NA, DA and plasma ESTI for 7 women during headout immersion.

Results are shown as mean ± SEM. They were obtained from the average of results between the follicular and luteal phases. Results of pre-immersion period were taken as 100 %.

Opposite effects of DA and NA on the renal handling of sodium suggest that relative concentrations of DA versus NA may be an important factor mediating the natriuretic response to certain physiological events (Krishna *et al* 1983). Thus, the ratio of DA to NA (DA/NA) was calculated and analysis of variance showed that there were significant changes in the ratio during the study period ($F=29.854$, $p< 0.0001$). Figure 5-2 shows the relative percentage changes of sodium excretion, DA/NA and plasma ESTI. The ratio doubled during the first hour of immersion, peaked at the second hour, and decreased to 175% at the third hour. It decreased further to 50% of the control value during the recovery period.

The correlations between the different variables were calculated for the results during the 3 hours of immersion period. Excretion of sodium did not correlate with urinary DA excretion or with plasma ESTI concentration. It correlated significantly with NA excretion and the ratio of DA/NA. Stepwise multiple regression showed that sodium excretion correlated significantly only to the DA/NA ratio ($\beta=-0.6360$, $p<0.02$). Plasma ESTI concentration correlated significantly with both DA excretion and DA/NA ratio. Stepwise multiple regression showed that plasma ESTI correlated with DA excretion ($\beta=0.5574$, $p<0.05$), but other variables did not account significantly for any further variation in ESTI.

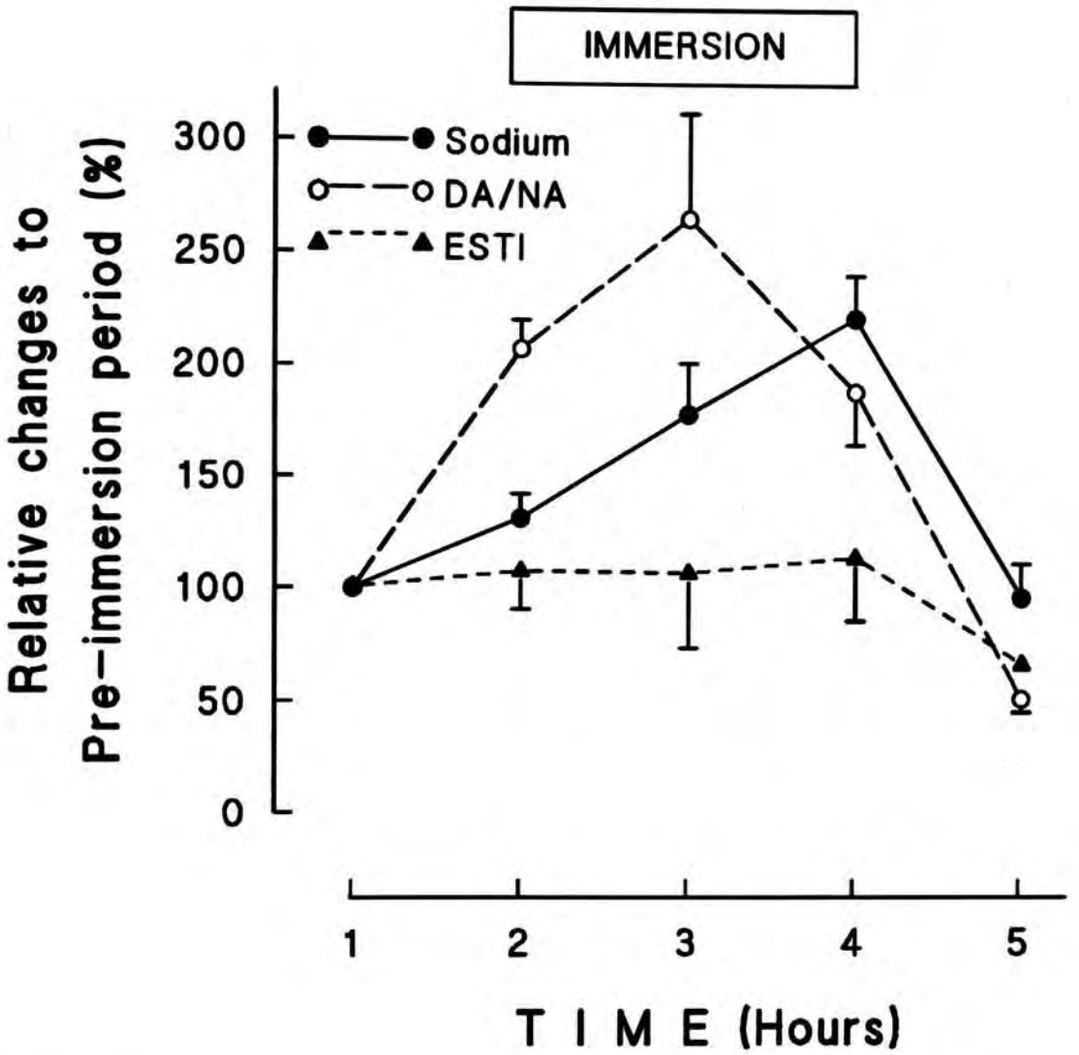


Figure 5-2: Changes in DA/NA ratio, plasma ESTI and sodium excretion for 7 women during headout immersion.

Results are shown as mean ± SEM. They were obtained from the average of results between the follicular and luteal phases. Results of pre-immersion period were taken as 100%.

DISCUSSION

A highly significant increase in fractional excretion of sodium during immersion indicates that the natriuresis is attributable primarily to decreased tubular reabsorption of sodium rather than to alterations in filtered sodium load (Epstein 1992). In a recent review, 8 different possible mechanisms have been listed for the decrease of sodium reabsorption (Epstein 1992). These mechanisms have been studied with variations in the experimental conditions. In the study designed to examine renal sodium handling during the follicular and luteal phases of the menstrual cycle in the present group of 7 women, it was shown that ANP increased 2-fold, and plasma renin activity and aldosterone concentration decreased by over 60% during the immersion period (Bisson *et al* 1992). Both ANP and the renin-aldosterone system are 2 of the mechanisms suggested to be responsible for natriuresis during immersion (Epstein 1992). The results presented here examine 3 other mechanisms on the list: the role of renal sympathetic nervous activity assessed by the excretion of free NA, the roles of free DA and the humoral natriuretic factor. This represents the largest number of mechanisms studied in a single group of subjects in one immersion study. The sodium status and the pattern of natriuresis during the 2 phases of the present group of subjects were not different (Bisson *et al* 1992). Pre-immersion urinary free NA, DA and plasma ESTI were also not significantly different. Results of the 2 phases were therefore averaged to reduce intra-individual variation.

In this study, the excretion of NA was suppressed by 50% throughout the course of immersion but during the post-immersion hour, NA excretion recovered by more than 180% compared to the pre-immersion level (Table 5-2 and Figure 5-1). The data clearly showed that the sympathetic nervous activity was depressed during water immersion. Recently, a similiar experimental design reported that suppression of urinary NA excretion was 30% in 10 healthy males throughout the course of the immersion (Grossman *et al* 1992). However, during the recovery post-immersion period, NA excretion was back to the baseline level. The difference in the magnitude of suppression during immersion may not be significant between the 2 studies. However, the increase in NA excretion during the post-immersion was much higher in

the present study. The significance of this phenomenon is not clear. It was speculated that it might be because the 2 populations studied were of different sexes. Sex-dependent variations in the excretion of urinary CATS during immersion have not been studied.

Urinary free NA reflected the circulating plasma NA concentration. However, previous immersion studies using plasma NA have shown conflicting results. There were studies showing no significant changes in plasma NA (Weihl *et al* 1981, Epstein *et al* 1983, Knight & Horvath 1987) and studies showing significant decrease (Krishna *et al* 1983, O'Hare *et al* 1986). No report showed an increase in plasma or urine NA levels during immersion. In one recent study, the measurement of cardiovascular variables such as central venous pressure, cardiac output, and stroke volumes were included in an immersion study, and plasma NA concentration was suppressed (Norsk *et al* 1990). The changes in measured cardiovascular variables suggest that the decrease in NA reflected a decrease in sympathetic nervous activity initiated by stimulation of low and high-pressure baroreceptors.

Suppression of plasma or urine NA concentrations during water immersion can only be used as an indirect measure of the renal sympathetic nervous activity. More direct indices, such as the measurement of renal venous NA levels, are required to delineate the effects of immersion on the kidney (Epstein 1992). The use of direct microneurographic recordings in renal nerves demonstrated reduced renal sympathetic nervous activity in dogs with natriuresis and diuresis during immersion (Miki *et al* 1989). In another animal study, NA was infused into cats to preinfusion levels and this abolished the renal responses to intravascular volume expansion (Rasmussen *et al* 1988). These results led to the conclusion that the reduction in renal sympathetic nervous activity plays an important role in the natriuresis in animals. Although the animal results may not be applicable to the human, a recent review on the subject summarized evidence to suggest suppression of renal sympathetic nervous activity is an important contribution to the natriuresis of immersion (Epstein 1992).

In this study, the excretion of free DA decreased over the period of infusion (Table 5-2 and Figure 5-1), but failed to reach statistical significance. Similar results were observed in a 4-h immersion study (Predel *et al* 1988). However, this finding did not agree with the result of a recent immersion study in which DA excretion exhibited a triphasic pattern (Grossman *et al* 1992). DA excretion was shown to decrease at the beginning of immersion, increased after the first hour, and significantly decreased during the recovery period. The experimental design of the 2 studies was similar. This discrepancy could be due to the difference in the magnitude of renal sympathetic nervous activity suppression, since the present group of women showed a comparatively higher suppression of NA excretion during the immersion period, which could facilitate natriuresis without an increase in DA. Furthermore, there was no correlation between sodium and DA excretion during immersion in this study.

DA has been considered a potentially important mediator of the natriuretic response to volume expansion (Epstein 1992). Dopaminergic blockage by domperidone and metoclopramide caused blunted natriuresis during immersion (Krishna *et al* 1985, Coruzzi *et al* 1989). The immersion-induced suppression of plasma aldosterone was prevented during metoclopramide administration but was unaffected by domperidone. It has been proposed that the DA system is important in natriuresis and aldosterone secretion during immersion (Crouzzi *et al* 1989). With dopaminergic blockage, there was a 6-8 fold increase in plasma prolactin level (Coruzzi *et al* 1989). Prolactin could attenuate natriuresis as it has been shown that prior administration of bovine prolactin results in a blunted natriuretic response to saline infusion (Lucci *et al* 1975).

Plasma DA concentration was reported to increase significantly during immersion (Krishna *et al* 1983). The physiological role of increased circulating DA was not clear. Furthermore, the excretion of urinary DA mainly reflected the renal synthesis, rather than the circulating plasma level (Lee 1993). However, this observation was not reproduced in another study (Grossman *et al* 1992).

It has been suggested that the DA/NA ratio may be an important factor mediating the natriuretic response to water immersion due to the opposite effects of NA and DA on

the renal handling of sodium. Plasma DA/NA ratio correlated significantly with natriuresis during water immersion and saline infusion (Krishna *et al* 1983). Urinary DA/NA ratio was reported to be significantly increased at the third hour of water immersion, but significantly decreased during the recovery period (Grossman *et al* 1992). In this study, the urinary DA/NA ratio was also calculated. The relative change of the ratio was plotted against the relative change of excretion of sodium and the plasma ESTI concentration (Figure 5-2). The ratio increased over 200% at the first hour of immersion, peaked in the second hour to over 250%, and remained at about 200% during the last hour of immersion. The ratio decreased significantly during the recovery phase, to 50% of the pre-immersion level. Sodium excretion correlated significantly with the DA/NA ratio in this study. Stepwise multiple regression analysis showed that there was an inverse relationship between this ratio and sodium excretion as the standardized coefficient of regression ($\beta=-0.6360$) was negative. This suggested that decrease in NA, i.e. reduced sympathetic nervous activity, was the driving force for sodium excretion during water immersion. The pattern of change was quite different from that reported by Grossman *et al* except during the recovery period (Grossman *et al* 1992). Difference in the pattern was expected as the changes of NA and DA excretion were different between the 2 studies.

The presence of a circulating natriuretic factor in volume expanded states has been documented (de Wardener & Clarkson 1985). Studies during the recovery hour following immersion revealed a continuing natriuresis despite the progressive volume contraction induced by the earlier period of immersion (Epstein 1992). It was shown that the continuous natriuresis during the recovery phase was not attributable to persistent aldosterone suppression (Epstein *et al* 1973). In this study, plasma ESTI concentration increased during the immersion hours but failed to reach statistical significance (Table 5-2, Figure 5-2). The increase was about 13% at the third hour. It would be of interest to see the changes if the immersion period was extended. However, the ESTI concentration decreased significantly to 66% of the pre-immersion level during the recovery period. The results suggest that plasma ESTI concentration was affected by physiological alterations in central blood volume in the

studied subjects. However, such a small increase indicates that ESTI does not play an important role in the natriuresis of immersion. If ESTI had to play a significant role, the natriuretic effect must be very potent. The presence of a urinary natriuretic factor and plasma sodium transport inhibitor in normal subjects undergoing immersion has been reported (Epstein 1976). A preliminary study also showed that plasma immunoreactive ouabain-like factor was increased during immersion and decreased promptly following cessation of immersion (Epstein 1992). The description of that preliminary result was similar to the findings of the present study.

It was also of interest to note that there was a significant correlation between the excretion of free DA and the plasma ESTI concentration during the course of immersion. This reflected similar responses of the 2 variables as shown on Figure 5-2. The physiological significance of this correlation is not clear.

This study showed that the relative change of NA and DA excretion was important in the natriuresis of immersion. Plasma ESTI concentration probably did not play an important role.

III. VOLUME EXPANSION BY SALINE INFUSION

MATERIALS AND METHODS

Four healthy Chinese male volunteers, aged 25 to 36 years, were recruited for the saline infusion study. They were on their usual diet and were not on any medication before the study.

After an overnight fast, the subjects arrived at the Clinical Pharmacology Study Unit at the Prince of Wales Hospital around 08:30 in the morning. Intravenous cannulae were inserted into both arms. One was for saline infusion and the other was for blood sampling. Heparin solution was used to keep the line open. The subjects emptied the bladder and the urine was discarded. They drank 200 ml of water and laid down on a bed at about 09:00. Subjects remained in the supine position for 30 min and a blood sample was taken. They remained supine throughout the study except to empty their bladder. Blood pressure was measured at 09:50. A urine sample was collected at 10:00 as the first hour urine sample. The same procedure was repeated at the next hour when a blood sample was collected at 10:30 and a second hour urine sample was collected at 11.00.

At 11:00, after the second set of samples, the intravenous saline infusion began. A 0.9% sodium chloride solution (153 mmol/l) was infused at 500 ml/h for 2 h. The blood and urine collections, and the blood pressure recordings were continued similarly to the first 2 hours. The saline infusion was stopped at 13:00, but the blood and urine collections, and monitoring of blood pressure, were continued for another 2 h. The study was completed at 15:00.

Urine samples were acidified for the measurement of urinary CATS as described in Chapter 3. Urinary electrolytes and creatinine were also measured as described in Chapter 4. Lithium heparin plasma was used to measure plasma ESTI concentration as described in Chapter 2. Plasma samples were also used to evaluate the renal

function tests and the total protein, albumin, and calcium on the Parallel Analytical System as described in Chapter 4. EDTA plasma was kept frozen at -70 °C for the measurement of renin activity. Serum samples were also kept frozen at -70 °C for the measurement of aldosterone.

Plasma renin activity was measured using a commercial kit, (Biodata Renin MAIA kit, Serono-Baker Diagnostics, Allentown, PA, USA). The kit was used according to the manufacturer's instructions. Plasma renin activity was measured as the angiotensin I liberated by renin. Briefly, plasma samples were incubated to generate angiotensin I at pH 7.4 at 37 °C for 3 h. An enzymatic inhibitor, phenylmethylsulphonyl fluorure, was added to prevent further enzymatic degradation of angiotensin I during the generation step. Afterwards, angiotensin I was measured by radioimmunoassay. Angiotensin I in samples or standards competed with iodinated angiotensin I for the binding sites on rabbit anti-angiotensin I antibody. Reaction mixtures were incubated at 4 °C overnight. After the incubation, the bound and free fractions were separated by adding sheep anti-rabbit γ-globulins coupled with magnetic particles. The application of a magnetic field allowed the sedimentation of the immunocomplexes. The supernatant was decanted and the radioactivity in the bound fraction was counted in a γ-counter. Cross reactivity of the anti-angiotensin I antibody is 0.06 % to angiotensin II and 0.02 % to angiotensin III. Sensitivity of the method is 0.1 ng/ml. The inter-assay precision CV was better than 10%.

Serum aldosterone was measured by a commercial solid-phase radioimmunoassay kit (Coat-A-Count® Aldosterone, Diagnostic Product Corporation, Los Angeles, CA, USA). The kit was designed to quantitate aldosterone levels in unextracted serum samples and it was used as described by the manufacturer. Briefly, serum samples and iodinated aldosterone tracer were added to polypropylene tubes coated with anti-aldosterone antibody and incubated overnight at 4 °C. The reaction mixture was aspirated and the tubes were counted in a γ-counter. Cross reactivity of the antibody is 0.06% to spironolactone, 0.033 % to 18-OH-corticosterone, 0.007 % to progesterone, 0.006 % to11-deoxycortisol, 0.002 % to corticosterone, less than 0.001 % to androsterone, cortisone, 11-dexamethasone, DHEA, prednisolone, and not

detected to androstendione, cortisol, estradiol, estriol, estrone, fludrocortisone, prednisone, pregnenolone, 17α-hydroxyprogesterone and testosterone. Detection limit of the method is 44 pM. The inter-assay precision CV was better than 10%.

Statistical analyses were as described in the previous section.

RESULTS

The results of plasma sodium, albumin-adjusted calcium and total protein concentrations are shown on Table 5-3. Significant changes were observed in plasma concentrations of sodium, calcium, total protein and albumin. Plasma sodium concentration increased by 2 mmol/l from the start of infusion and remained elevated. The albumin-adjusted calcium concentration did not show any significant change during infusion and recovery periods. Total protein concentration decreased during the infusion period and remained lower than the baseline level at the end of the recovery period. Mean relative changes of total protein concentration showed that there was a 10% decrease in concentration at the second hour of infusion period and a 6% decrease in concentration at the second hour of recovery period. These changes were used to show that an average of 10% expansion of the extracellular fluid volume was achieved by the saline infusion procedure.

Table 5-4 summarises the results for urine flow rate, sodium excretion and GFR. One-way ANOVA showed that there were significant changes in the urine flowrate and the excretion of sodium. Urine flowrate increased during the second hour of the baseline period, probably due to the initial drinking of 200 ml of water leading to a diuresis. However, the urine flowrate decreased during the course of saline infusion and the subsequent recovery period. Excretion of sodium increased during saline infusion and the subsequent recovery period. Using the average values of 2 initial baseline results as the background sodium excretion, the mean cumulative sodium excreted during saline infusion and the subsequent recovery period was 19%(range 14-24%) of the infused sodium load. There was a tendency for the GFR to decrease during the 2 hours of saline infusion, but it did not reach statistical significance.

Table 5-3: **Plasma sodium, albumin-adjusted calcium, and total protein concentration data for the 4 subjects undergoing saline-infusion.**

Results are expressed as mean ± SEM

Time (h)	Sodium (mmol/l)	Albumin-adjusted Calcium (mmol/l)	Total Protein (g/l)
Baseline - 1	136 ± 2	2.10 ± 0.04	74 ± 1
Baseline - 2	136 ± 1	2.15 ± 0.04	76 ± 1
Infusion - 1	138 ± 1	2.14 ± 0.05	76 ± 2
Infusion - 2	138 ± 1	2.15 ± 0.01	69 ± 1
Post-infusion - 1	138 ± 1	2.06 ± 0.02	70 ± 1
Post-infusion -2	138 ± 1	2.13 ± 0.04	71 ± 1
ANOVA			
F-Ratio	6.0000	1.9659	10.474
p	0.0030	0.1426	0.0002

Table 5-4: **Urine flow, sodium excretion, and GFR data for the 4 subjects undergoing saline infusion.**

Results are expressed as mean ± SEM

Time (h)	Urine Flowrate (ml/ min)	Sodium excretion (μmol/min)	G F R (ml/min)
Baseline - 1	3.93 ± 0.98	187 ± 52	130 ± 13
Baseline - 2	5.60 ± 1.06	176 ± 48	130 ± 9
Infusion - 1	2.26 ± 0.50	224 ± 46	118 ± 4
Infusion - 2	2.46 ± 0.41	322 ± 44	128 ± 7
Post-infusion - 1	2.07 ± 0.26	350 ± 33	131 ± 10
Post-infusion -2	1.56 ± 0.27	318 ± 38	133 ± 8
ANOVA			
F-Ratio	9.2497	31.029	1.0768
p	0.0004	< 0.0001	0.4119

As the baseline value for sodium excretion varied between the 4 subjects the relative changes of sodium excretion were calculated. The pattern of relative changes is shown on Figure 5-3. There was a significant difference between the baseline, infusion and recovery periods ($F=6.9104$, $p=0.0040$). Maximum excretion was observed during the first hour of recovery, reaching 227% of the baseline value.

Table 5-5 shows the results for excretion of CATS. ANOVA showed that there were no statisticallly significant differences between the 6 hours of study for the 3 urinary CATS. Since there were large variations in the excretion of urinary free CATS between the subjects, relative changes of each CATS were calculated. The patterns of relative changes of urinary free CATS and the DA/NA ratio are shown on Figure 5-3. The ANOVA tests on the relative changes of CATS excretion did not show any significant changes. NA excretion decreased by 16% during second hour of infusion and the first hour of recovery, but increased to 90% of the baseline level during the second hour of recovery. Excretion of A remained elevated during the infusion and recovery periods, reaching 32% higher than the baseline level during the recovery period. Excretion of DA decreased by 8% during the first hour of infusion, but returned to baseline levels during the remaining time of the study. The DA/NA ratio and relative changes of the ratio were also calculated. The ratio increased during the infusion and remained elevated at the end of the recovery period. However, the changes were not statistically significant by 1-way ANOVA.

Changes in plasma ESTI , plasma renin activity and serum aldosterone concentrations are summarized on Table 5-6 and the patterns of relative change are shown in Figure 5-4. Renin and aldosterone were suppressed till the second hour of the recovery period. Suppression of aldosterone was more severe than that of renin at the last hour of observation. Aldosterone was only 38.5% of the baseline level and renin was 50.5% of the baseline level. Plasma ESTI increased during the first hour of infusion and then decreased by 10% during the remaining observation period, but these changes were not statically significant.

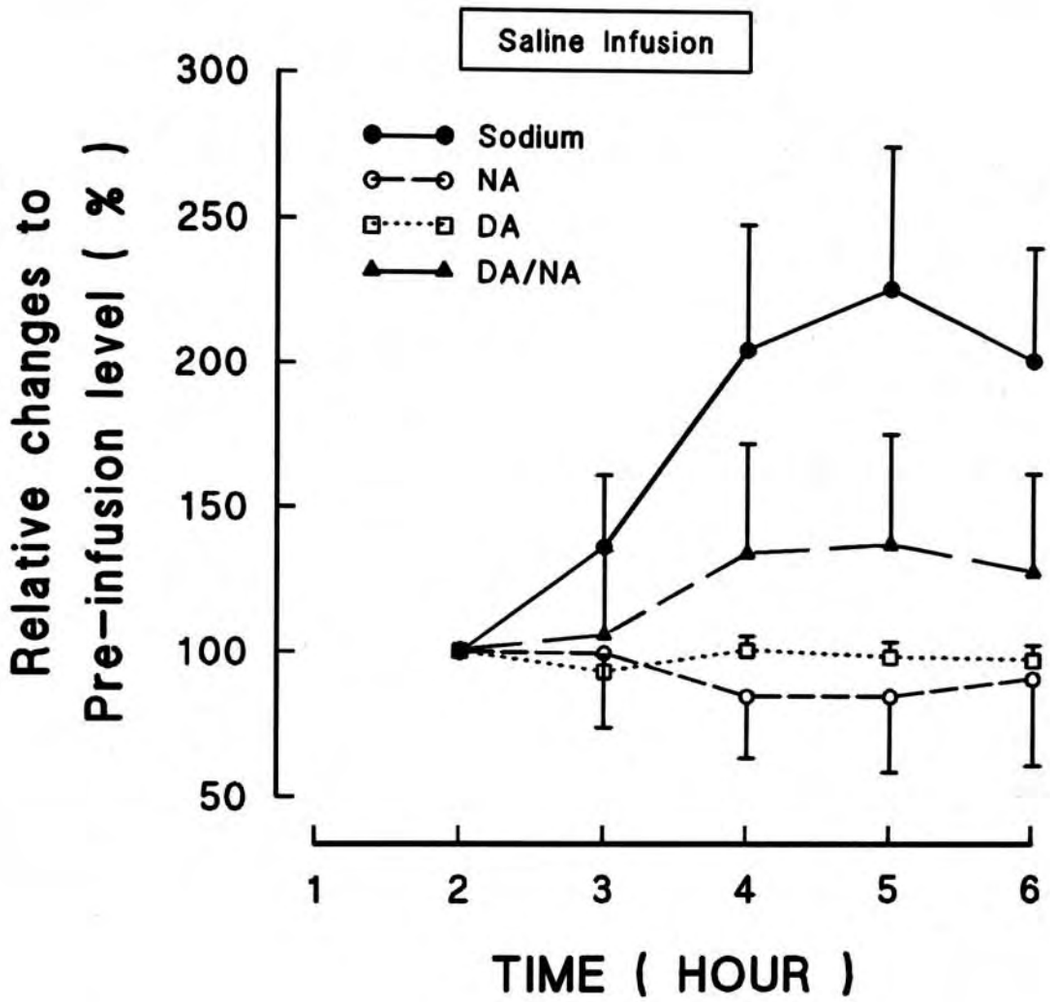


Figure 5-3: **Relative changes of urinary free CATS, DA/NA ratios and sodium excretion during saline infusion.**

Table 5-5: Urinary excretion of CATS in the 4 subjects undergoing saline infusion.

Results are expressed as mean ± SEM
Noradrenaline (NA), Adrenaline (A), Dopamine (DA)

Time (h)	NA excretion (pmol/min)	A excretion (pmol/min)	DA excretion (pmol/min)
Baseline - 1	155 ± 35	68 ± 36	1382 ± 97
Baseline - 2	137 ± 64	66 ± 31	1326 ± 67
Infusion - 1	116 ± 25	56 ± 19	1250 ± 49
Infusion - 2	100 ± 24	49 ± 11	1351 ± 41
Post-infusion - 1	96 ± 21	57 ± 10	1318 ± 38
Post-infusion -2	99 ± 15	57 ± 9	1305 ± 45
ANOVA			
F-Ratio	1.2644	0.3465	0.6565
p	0.3294	0.8732	0.6615

Table 5-6: Plasma ESTI, renin activity and serum aldosterone concentration data for the 4 subjects undergoing saline infusion.

Results are expressed as mean ± SEM

Time (h)	Plasma ESTI (nM OE)	Plasma Renin (ng/ml/h)	Serum Aldosterone (pM)
Baseline - 1	9.9 ± 3.53	0.58 ± 0.15	483 ± 143
Baseline - 2	15.6 ± 3.58	0.60 ± 0.10	294 ± 81
Infusion - 1	15.9 ± 1.98	0.55 ± 0.05	279 ± 94
Infusion - 2	13.3 ± 1.11	0.37 ± 0.05	184 ± 55
Post-infusion - 1	13.3 ± 2.27	0.27 ± 0.01	141 ± 39
Post-infusion -2	13.6 ± 1.23	0.28 ± 0.02	138 ± 25
ANOVA			
F-Ratio	1.0351	4.1665	3.4951
p	0.4327	0.0142	0.0271

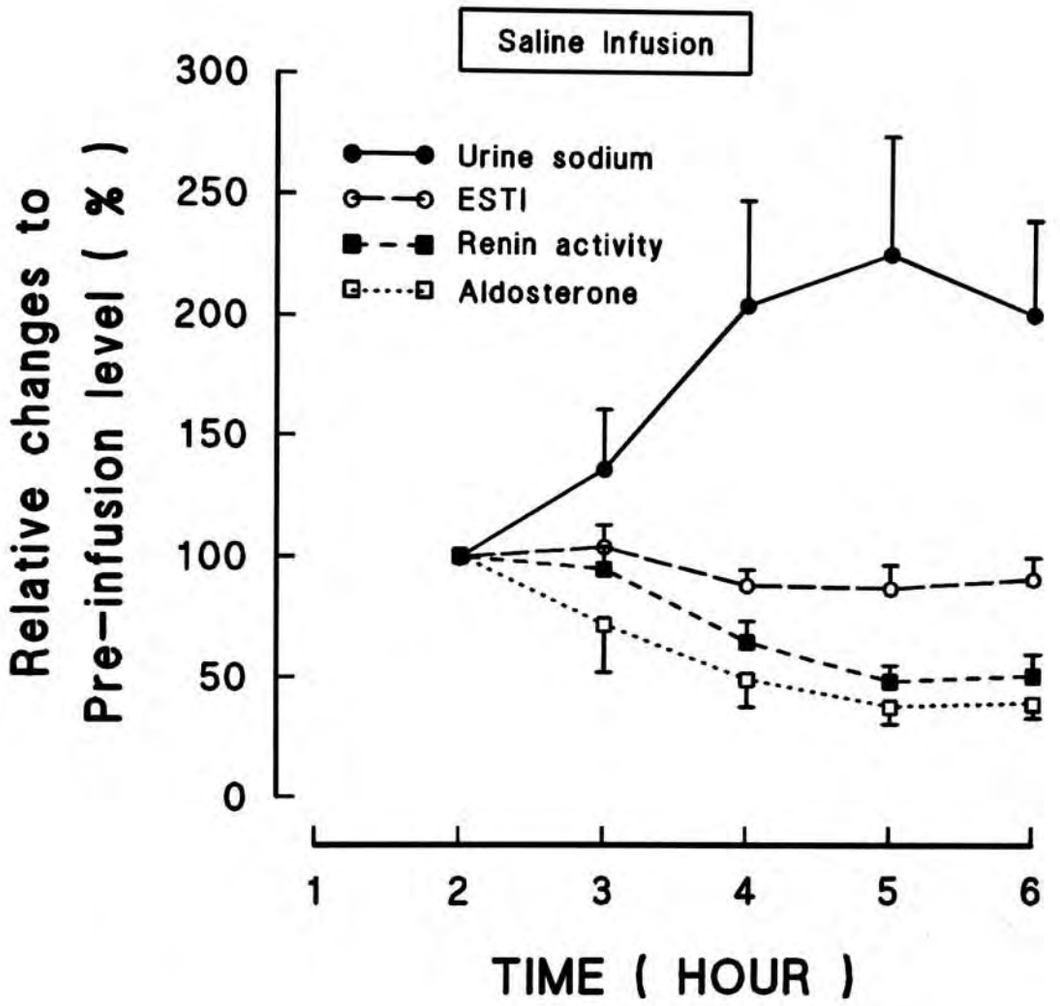


Figure 5-4: **Relative changes of plasma renin, ESTI, and serum aldosterone levels and sodium excretion during saline infusion.**

To understand the contributing factors for natriuresis during saline infusion, the data of the infusion and recovery periods were used for a correlation study. The recovery period was also included as 80% of the infused sodium load was retained in the body at the end of the recovery period. Sodium excretion was significantly correlated with urine flowrate ($r=0.527$, $p=0.0361$), GFR ($r=-0.572$, $p=0.020$), DA excretion ($r=0.874$, $p<0.0001$), renin activity ($r=-0.716$, $p=0.0018$) and aldosterone concentration ($r=-0.687$, $p=0.0033$). Stepwise multiple regression analysis showed sodium excretion was only correlated positively with DA excretion ($\beta=0.763$, $p<0.0001$) and inversely with plasma renin activity ($\beta=-0.434$, $p=0.0002$). The correlation of sodium excretion with DA excretion is shown on Figure 5-5.

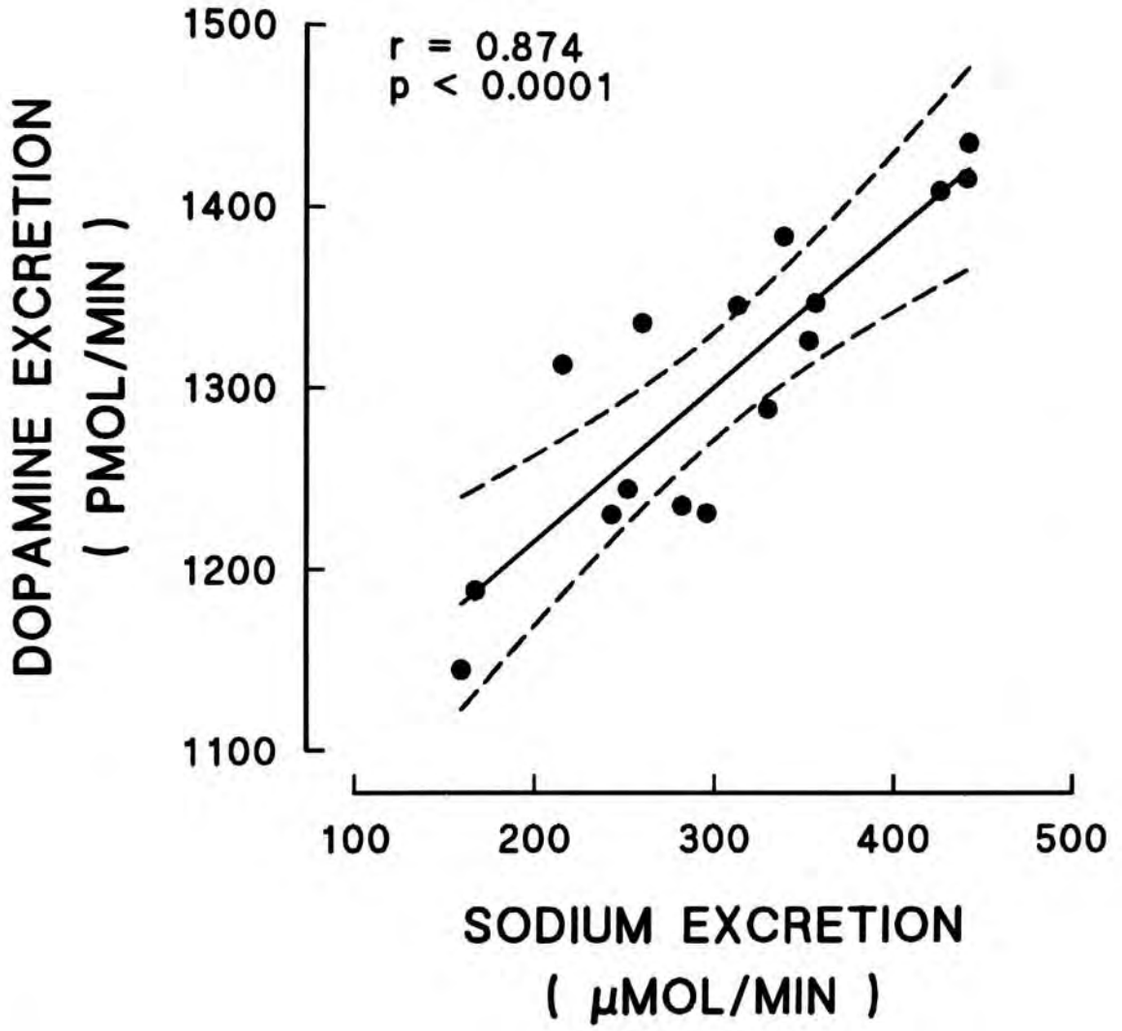


Figure 5-5: Correlation of sodium excretion and DA excretion.

DISCUSSION

Acute intravenous infusion of isotonic saline in man has been widely used to study the intra- and extra-renal factors that can modulate natriuresis. Results are often conflicting as the procedures for infusion are not standardized. The volume of isotonic saline infused ranged from 1 liter (Herlitz *et al* 1990) to over 4 liters (Jeffrey *et al* 1989) and the duration of infusion ranged from 10 min (Widgren *et al* 1991) to 4 h (Tulassay *et al* 1988). These maneuvers affect the degree of volume expansion and may trigger different physiological mechanisms. For example, most saline infusion studies reported significant increases in plasma ANP, however an infusion of 22 ml/kg of saline over 20 min failed to show an increase in ANP (Drummer *et al* 1992). This makes comparison of data between studies difficult. In the present study 1 liter of isotonic saline was infused over a period of 2 h. Significant volume expansion was achieved as indicated by the 10% decrease in the plasma total protein concentration during the second hour of infusion (Table 5-3) and it was 6% lower than the baseline level at the end of the recovery period. A similar combination of conditions to study the contributions of urinary free DA and plasma ESTI in natriuresis has not been reported in the literature.

In the present study, the subjects drank 200 ml of water at the beginning of the 2 h baseline period. An increase in urine flowrate was observed, probably as a result of this initial water intake. However, the urine flowrate decreased significantly after starting the saline infusion (Table 5-4). The average total urine output for the last 4 h of the experiment for the 4 subjects was about 50% of the infused volume of saline. In many studies reported in the literature water intake was allowed during saline infusion to ensure adequate urine flow (Singer *et al* 1987, Jeffrey *et al* 1989, Stenvinkel *et al* 1992) and the urine flow rate did not significantly change during infusion and recovery periods. In studies that did not provide additional water intake during infusion, urine flow rate decreased significantly (Alexander *et al* 1974, Tulassay *et al* 1988). Saline infusion increased the plasma osmolarity as shown by a significant increase in plasma sodium concentration (Table 5-3). Osmolarity is an important modulator of antidiuretic hormone secretion. An increase in osmolarity

would cause an increase in the antidiuretic hormone concentrations and a decrease in the urine output. When isoosmotic glucose is infused there is a significant increase in the urine flow without affecting the excretion of DA (Stenvinkel *et al* 1992). However, it has not been shown whether a decrease in urine flow rate can affect excretion of DA.

In the present study, significant natriuresis was observed in all the subjects. Sodium excretion was over 200% of baseline at the second hour of infusion and peaked at the first hour of the recovery period to 230% of the baseline period (Figure 5-3). The relative increase in sodium excretion appears to be related to the amount of sodium infused. Infusion of more than 300 mmol sodium led to a 3 to 4-fold increase in sodium excretion (Alexander *et al* 1974, Singer *et al* 1987, Tulassay *et al* 1988). A higher sodium load of more than 600 mmol was reported to cause a 5-fold increase in sodium excretion (Jeffery *et al* 1989). Since only 154 mmol of sodium was infused in the present study, a 2-fold increase in sodium excretion was appropriate. Most of the studies also observed that peak excretion of sodium occurred within an hour after the infusion and the plasma ANP response followed that of sodium (Singer *et al* 1987, Tulassay *et al* 1988, Jeffery *et al* 1989). This observation led to investigation of the contribution of other factors, such as DA and plasma ESTI, to the natriuresis in saline infusion.

It is generally believed that the GFR is stable or will increase following ECF volume expansion with saline (Burg 1981). In this experiment, GFR was estimated by the creatinine clearance. It was observed that GFR decreased by 9% during the first hour of infusion and returned gradually to 2% above the baseline value at the end of the observation periods. However, the changes did not reach statistical significance. Despite a 4-fold higher sodium load during the infusion, a similar pattern was observed in another study using creatinine clearance for the estimate of GFR (Jeffery *et al* 1989). The changes also did not reach statistical significance. Using the inulin clearance to measure GFR, a significant decrease of GFR was observed with saline infusion, but not during water diuresis or infusion of isoosmotic glucose solution (Stenvinkel *et al* 1992). Another study using ^{51}Cr-EDTA clearance also reported a

significant fall in GFR in healthy controls and unilaterally nephrectomized humans following saline infusion (Sørensen *et al* 1987). The accuracy of the measurement of GFR by creatinine clearance is inferior to that of the inulin or ^{51}Cr-EDTA clearance methods and this may explain the lack of the significant decrease of GFR in the present study.

Both plasma renin activity and serum aldosterone concentration decreased during infusion and remained suppressed at the recovery period (Table 5-6 and Figure 5-4). This pattern is consistent with other reports in the literature (Krishna *et al* 1983, Singer *et al* 1987, Stenvinkel *et al* 1992). The renin-angiotensin system is important in the physiologic regulation of fluid and electrolyte balance (Rabkin & Dahl 1993). In this study, both renin and aldosterone were among factors significantly correlated with the excretion of sodium during and after the saline infusion. However, only DA excretion and plasma renin activity showed significant correlation with sodium excretion in stepwise multiple regression analysis.

The role of urinary free DA excretion in the natriuresis of saline infusion has been conflicting. In the present study, there was an initial fall of 8% in DA excretion at the first hour of the infusion period and then it rose back to the baseline level throughout the study periods (Table 5-5 and Figure 5-3). These changes were not significant. There was large variation in baseline DA excretion and the response to saline infusion also varied widely. With the small number of subjects involved in the study, large relative changes are required to reach statistical significance. The excretion of DA and sodium were significantly correlated during the study period (Figure 5-5).

Isotonic saline infusion by 600 mmol of sodium over a period of 4 h did not show a significant rise in urinary free DA excretion in 9 healthy male subjects (Jeffery *et al* 1989). However, during the recovery period, urinary DA increased significantly by 26% compared to the pre-infusion level. A 5-fold increase in sodium excretion was reported and the increase in DA excretion was comparatively small. When the same subjects were given carbidopa to inhibit intrarenal production of DA before a second saline infusion, sodium excretion was not affected. The results showed no evidence of

a facilitating role for DA in the natriuretic response to saline infusion. The authors also noted that less than 30% of the sodium was excreted during the observation periods. They suggested that DA may assume greater significance during the later stages of the response.

On the other hand, there are other studies which show a significant increase in DA excretion during saline infusion. Alexander *et al* (1974) reported a 3.5-fold increase in sodium excretion and a significant 28% increase in DA excretion after 3 h saline infusion in 7 women. The subjects were kept on a 59 mmol/l sodium diet for 3 days before the infusion. Castellano *et al* (1986) reported an 18% increase in 24-h DA excretion in a group of 8 healthy subjects after a 2-h saline infusion. They were kept on a diet containing 120 mmol/d sodium and 80 mmol/d potassium for 5 d before the infusion. Two other studies from the same research group reported less than 20% but significant increase in DA excretion during the second hour of the infusion and the recovery periods (Stenvinkel *et al* 1992, Stenvinkel *et al* 1992a). Both studies showed that the subjects had more than 2 fold increase in sodium excretion. The DA response to the large increase in sodium excretion in human studies was comparatively small.

DA excretion in saline infusion was more exaggerated in animal models. There was a 40% increase in rats (Hansell *et al* 1988) and 300% increase in dogs (Sowers *et al* 1984). A significant increase in DA excretion in these 2 animal studies was not observed during the first hour of saline infusion. In rats, the relative contribution of DA excretion to the natriuretic response of saline infusion was related to the degree of volume expansion (Chen & Lokhandwala 1991). DA appears to play a greater role in the overall natriuresis response during modest degrees of volume expansion (2.5-5.0% increase in body weight). Another study also concluded that endogenous DA contributes to the natriuresis of saline infusion by small, but not large, increases in extracellular fluid volume in anaesthetized rats (Bass & Murphy 1991). Validation of this observation in human studies has not been reported.

The use of DA receptor blockade suggests that renal DA may play a significant role in the natriuresis of saline infusion. Administration of metoclopramide, blocking mainly DA_2 receptors, in 2 different human studies decreased the magnitude of the saline infusion induced natriuresis, but did not completely abolish it (Krishna *et al* 1985, Tulassay *et al* 1988). The results suggested that in acute volume expansion, natriuresis is mediated only in part by DA (Tulassay *et al* 1988). Furthermore, metoclopramide prevented the suppression of plasma aldosterone and thus, may play a critical role in mediating the aldosterone response in volume expansion (Krishna *et al* 1985). On the other hand, administration of carbidopa, an inhibitor of dopa decarboxylase, in 9 males failed to suppress sodium excretion significantly indicating that renal DA does not play an important role in natriuresis of saline infusion (Jeffery *et al* 1989). These few reports in human studies do not support a major role of DA in saline infusion to facilitate natriuresis.

In the rat, the natriuresis associated with modest (1.7 to 3.4% body weight), moderate (6.3% body weight) and large (13% body weight) increments in extracellular fluid volume with either saline or Ringer's lactate solution was attenuated by selective and nonselective DA receptor antagonists (Bass & Murphy 1990). The effect of DA receptor blockade was also reported to be dependent on the degree of hypervolemia (Hansell & Fasching 1991). The DA system appears to be relatively more important in promoting natriuresis at the lower physiological range of hypervolemia, and other factors have a greater impact at the high range. Carbidopa was also reported to attenuate natriuresis produced by saline infusion in conscious dogs (Sowers *et al* 1984). On the other hand, Cuche *et al* (1983) showed that infusion of hypoosmotic saline into anesthetized dogs caused a large natriuresis without a change in DA excretion. DA excretion did increase during infusion with isoosmotic and hyperosmotic saline. The authors concluded that the dissociation between sodium and DA excretion does not support a physiological role in renal handling of sodium during volume expansion. It has also been reported that neither DA_1 receptor blockade with SCH 23390 nor DA_2 receptor blockade with domperidone can demonstrate any effect on the natriuresis accompanying extracellular volume expansion with isoosmotic saline (Bass & Murphy 1990). A recent report where

saline was infused at a very slow flowrate directly into the renal artery concluded that renal DA is not directly responsible for the observed natriuresis (Bullivant & Muñoz 1993). Thus, renal DA may not play a significant role in saline infusion natriuresis even in animal studies. More studies to understand the conflicting results are necessary.

Urinary NA excretion in this study decreased by 16% at the second hour of infusion and remained suppressed during the first hour of recovery period. It rose back to 90% of the baseline level at the last hour of the recovery period. However, these changes were not statistically significant by ANOVA (Table 5-5 and Figure 5-3). Although the urinary DA/NA ratio increased by 30% after the first hour of the infusion, the increase did not reach statistical significance. The results indicated that there was no significant decrease in the renal sympathetic nervous activity as observed during water immersion as described in the previous section. Excretion of NA was reported to decrease or remain unchanged during saline infusion in both human and animal studies (Alexander *et al* 1974, Cuche *et al* 1983, Sowers *et al* 1984, Castellano *et al* 1986, Hansell *et al* 1988, Stenvinkel *et al* 1992). Most studies have reported a decrease in plasma NA during the infusion and the recovery period (Cuche *et al* 1983, Krishna *et al* 1983, Tulassay *et al* 1988, Widgren *et al* 1991, Stenvinkel *et al* 1992). The decrease in plasma NA concentration could be caused by the dilution of the plasma instead of a decrease of the renal sympathetic nervous activity. It has also been reported that renal nervous activity may not be a major factor for controlling renal excretory function during either hypovolemia or hypervolemia in an animal study (Peterson *et al* 1984). Krishna *et al* (1983) reported the plasma DA/NA ratio was significantly correlated with the sodium excretion. However, there was no correlation of this ratio with sodium excretion in the present study.

Plasma ESTI concentration did not decrease significantly during the infusion and recovery periods in this study (Table 5-6 and Figure 5-4). The decrease in ESTI could reflect a dilution of plasma volume as the magnitude of decrease was similar to that of the total protein and as plasma ESTI is tightly bound to proteins. The results were consistent with de Wardener's hypothesis that a normal intact kidney should

have the ability to excrete additional sodium load. Other mechanisms were operating to excrete the sodium challenge, for example, the renin-angiotensin system. Recently, it was reported that plasma Na,K-ATPase inhibitory activity remained unchanged in a group of salt-resistant borderline hypertensives undergoing saline infusion (Borghi *et al* 1992). On the other hand, plasma Na,K-ATPase inhibitory activity in a group of salt-sensitive borderline hypertensives increased during the second hour of saline infusion. The salt-sensitive group also showed a delayed ANP response to saline infusion. It was suggested that the increase of plasma ESTI in the salt-sensitive group was due to the lack of ANP response. ANP has been reported to inhibit the release of sodium pump inhibitor in vivo in man (Crabos *et al* 1988, Borghi *et al* 1991).

Increase in plasma ESTI after saline infusion has been reported in some studies in man and animals (Gonick *et al* 1977, Pamnani *et al* 1978, Poston *et al* 1982, Tamura *et al* 1987, Hamlyn *et al* 1988). A 4 fold increase in serum inhibitory action after saline-infusion was reported in rats (Gonick *et al* 1977). Pamnani *et al* (1978) using a vascular transport bioassay showed that circulating inhibitors were modestly increased in response to saline infusion. Poston *et al* (1982) showed that urine extracts from saline infused subjects caused a significant fall in the total sodium efflux rate constant in a leucocyte preparation. However, the inhibition may not be specific to the Na,K-ATPase activity as both the ouabain-sensitive and ouabain-insensitive components of the rate constant were affected. Lysophosphatidylcholines were identified as one of the plasma ESTI in saline infused pigs (Tamura *et al* 1987). Hamlyn *et al* (1988) showed an average of 14% reduction in sodium pump activity after saline infusion in rats, pigs, sheep and man.

In summary, the present saline infusion study showed a significant increase in sodium excretion and significant depressions in renin and aldosterone. Both excretion of urinary free DA and the plasma ESTI appeared not to be major facilitators for the natriuresis induced by saline infusion. Measurement of these 2 factors should be continued for several hours as 80% of the sodium load had not been excreted at the end of the study.

IV. ORAL SALT LOADING ON A FREE DIET

MATERIAL AND METHODS

Five normotensive, ambulatory, healthy Chinese subjects (4 males and 1 female, aged 23 to 35 years) without a family history of hypertension were studied. All participants gave informed consent. During the nine day study period, alcohol and medications were avoided. From day 1-3, all subjects were on a diet of 70 mmol sodium per day (low salt diet) provided by the hospital kitchen. From day 4-6, they were on their normal diet plus 200 mmol of sodium supplement. They took 10 "Slow Sodium" tablets (Ciba Laboratories, West Sussex, UK) at 9:00 am and another 10 tablets at 1 pm (high salt diet). From day 7-9, they were on their normal diet with unrestricted sodium intake (free diet).

At the end of the low salt diet (morning of day 4), subjects were weighed and their blood pressure measured at 8 am before breakfast and after sitting for 5 min, using a Hawksley random-zero sphygmomanometer. Mean arterial pressure (MAP) was calculated as described in Chapter 4. Venous blood samples were collected for the measurement of plasma ESTI, sodium, potassium, creatinine, total protein and albumin. At the end of the high salt diet (morning of day 7), body weight, blood pressure and venous blood samples were similarly taken.

Throughout the study period, subjects collected their daily urine samples into 2 plastic bottles containing 25 ml of 5 M HCl. The first bottle was used to collect urine from 8 am to 10 pm (the day collection), and the second bottle was for the collection between 10 pm to 8 am (the night collection). Aliquots of the urine samples were frozen at -20 °C until assayed for urinary free CATS, sodium, potassium and creatinine. 24-hour urinary excretion of these measured analytes were calculated as the sum of the excretion from the day and night collections.

Plasma ESTI was measured as described in Chapter 2. Other plasma variables were measured on the Parallel Analytical System as previously described in this Chapter. Urinary sodium, potassium and creatinine were measured on the Astra 8 analyser as previously described in Chapter 4. Urinary free CATS were measured as described in Chapter 3.

All data were expressed as mean ± SEM. For plasma results, the Wilcoxon signed rank test was used to determine the significance of the difference between the low salt and high salt diets. The urine collections on the last day of the low salt diet were chosen as the baseline for comparison. Changes in urinary measurements were analysed with respect to time using ANOVA for repeated measurement. There were large inter-individual variations in the urinary excretion of the measured analytes. Thus, relative % changes of sodium, DA, NA and DA/NA ratio were calculated. Excretion at the end of the low salt diet were taken as 100%. Correlation coefficients between the different measurements were determined by simple regression analyses. P values of less than 0.05 were considered statistically significant.

RESULTS

Plasma results

Table 5-7 shows the effect of oral salt loading on blood pressure, body weight, plasma concentrations of the different measured parameters. The 5 subjects showed changes in their MAP from -17.2 to 1.6% but the overall mean was not significantly different from the baseline. There was a significant decrease in diastolic BP during the high salt diet. There was a significant increase in body weight (mean 1.0%, $p<0.05$, range 0.3-2.8%) after high salt intake for 3 days. Both plasma total protein and albumin showed a significant decrease in concentration. However, there was no significant change in plasma ESTI concentration.

Urine sodium excretion

Figure 5-6 shows the sodium excretion for the day, night and 24-hour collections. ANOVA showed that there were significant differences in sodium excretion for all the 3 collections ($F=17.24$, $p<0.0001$ for the 24-hour collection; $F=11.87$, $p<0.0001$ for the day collection; $F=7.34$, $p < 0.0001$ for the night collection). During the first 3 days of low salt diet, sodium excretion decreased to 40 mmol/d. On day 4, the subjects were on their own free diets plus 200 mmol of sodium supplementation. The sodium excretion increased and peaked on day 6 to 390 mmol/d. On day 7, the supplementation was stopped and the subjects were on their own free diets. Sodium excretion decreased gradually and reached 190 mmol/d on day 9. Using the Scheffe test, sodium excretion on days 5 - 7 were statistically higher than that on day 3. For the day urine collection, sodium excretion peaked on day 5 and decreased slightly on day 6. On the other hand, sodium excretion continued to increase to day 6 during the night collection. About 70% of the sodium load was excreted during the day collection period. However, on day 4, the day immediately after the low salt diet, only 50% of the sodium load was excreted during the day collection.

Table 5-7: Effect of oral sodium intake on blood pressure, body weight, plasma ESTI, and other plasma variables.

	Low salt diet	High salt diet
Blood pressure (mmHg)		
Systolic	98 ± 2	100 ± 3
Diastolic	67 ± 4	59 ± 3 *
Mean arterial	78 ± 2	73 ± 3
Body weight (kg)	56.1 ± 2.7	56.7 ± 2.7 *
Sodium (mmol/l)	137 ± 0.7	137 ± 0.4
Potassium (mmol/l)	3.7 ± 0.1	3.5 ± 0.1
Creatinine (μmol/l)	90 ± 4	85 ± 3
Total protein (g/l)	82 ± 2	75 ± 2 *
Albumin (g/l)	49 ± 1	45 ± 1 *
Na,K-ATPase inhibitor (nmol OE/l)	14.1 ± 2.1	14.1 ± 1.7
DLI (μg/l)	1.24 ± 0.04	1.21 ± 0.17

* denotes $p<0.05$ by Wilcoxon signed-rank test when compared with low salt diet.

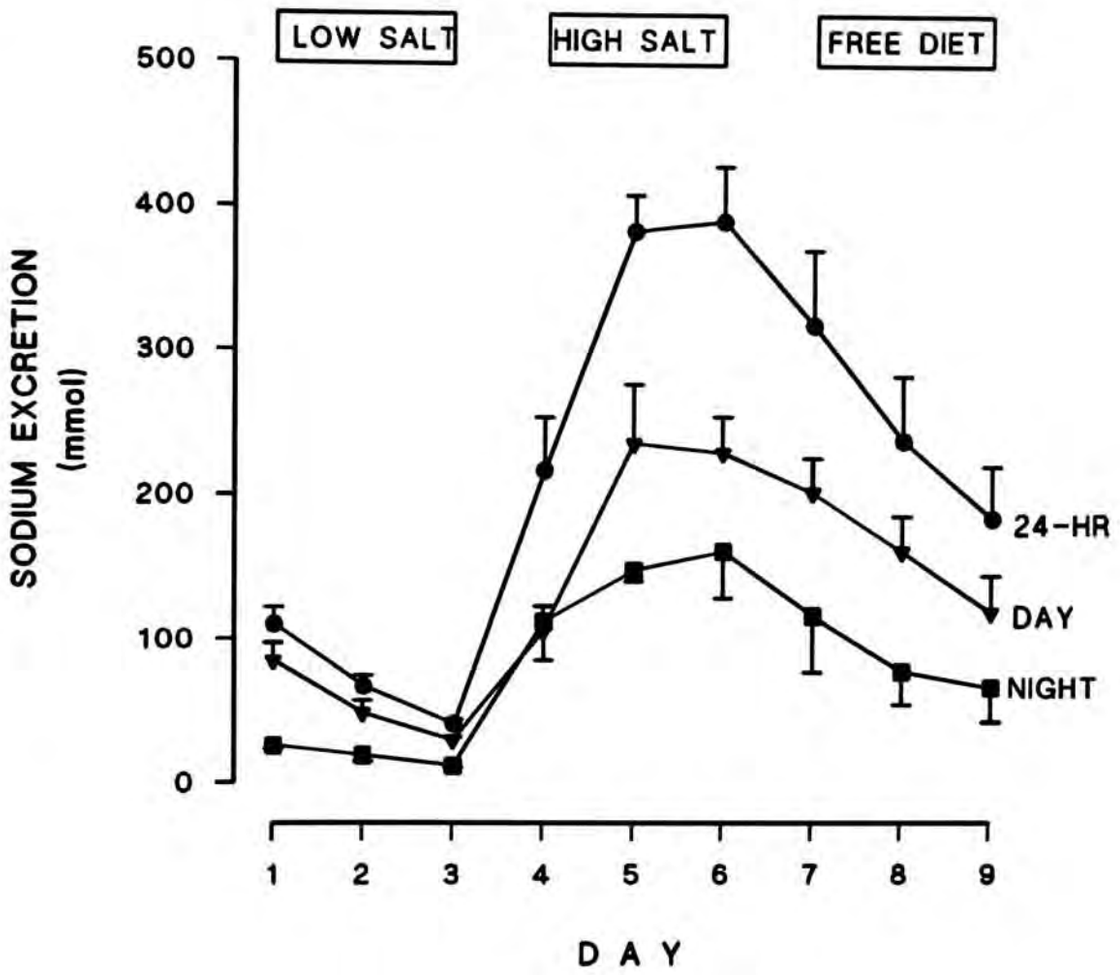


Figure 5-6: Excretion of sodium during the low salt, high salt and free diet periods.

Low salt: low salt diet provided by the hospital kitchen for days 1-3.
High salt: free diet supplemented with 200 mmol sodium (20 Slow Sodium tablets taken twice daily) for days 4-6.
Free diet: free diet for days 7-9.

Urine DA excretion

Figure 5-7 shows the DA excretion for the day, night, and 24-hour collections for the 9-day study period. There were no significant changes in DA excretion in any of the collections. Comparing the excretion between the day and night for each day, only on day 4 was there a significant difference. On day 4, DA excretion was highest during the day collection, but lowest during the night collection when compared to all the other collections during the study. On this day, DA excretion increased as the sodium increased during the day; however, DA excretion showed a paradoxical drop when the sodium excretion continued to rise during the night period.

Figures 5-8, 5-9, and 5-10 show the relative % changes of DA excretion for the day, night and 24-hour collections, respectively. There were no significant changes of DA excretion. On day 4, the relative %change of DA excretion for the day collection was still the highest (Figure 5-8) and that for the night collection was also the lowest (Figure 5-9).

Urine NA excretion

Figure 5-11 shows the NA excretion for the day, night and 24-hour collections. For the 24-hour collection, NA excretion showed an inverse relationship with the sodium excretion. It increased from day 1 to day 4 during the low salt period, decreased sharply during the sodium supplementation, and then gradually increased during the free diet period. However, these changes did not reach statistical significance. For the day collection, NA excretion peaked on day 4 and was lowest on day 6. For the night collection, NA excretion showed relatively smaller changes. It increased slightly during the low salt period, decreased on day 4, and continued to increase from day 5 to day 9. When the relative % changes in NA excretion were calculated, there were still no significant changes by ANOVA.

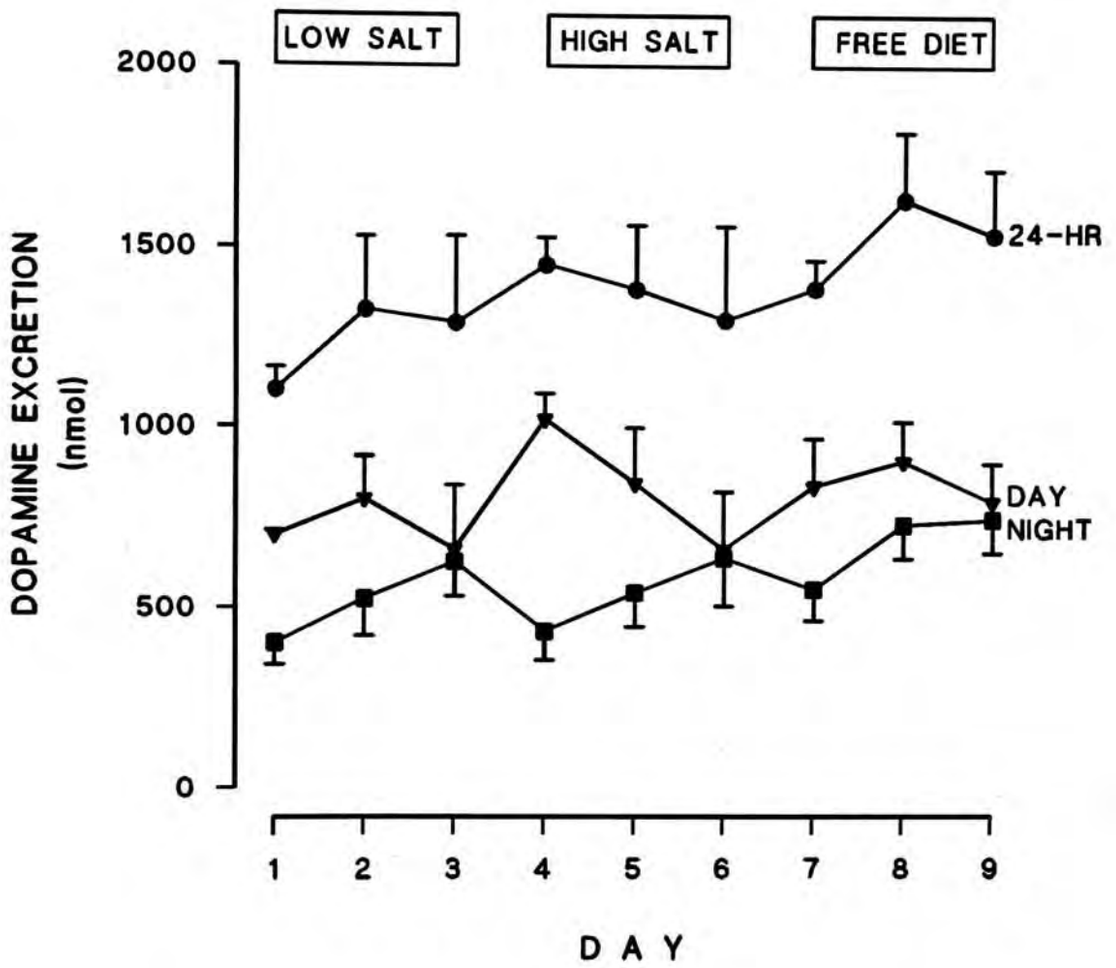


Figure 5-7: Excretion of DA during the low salt, high salt and free diet periods.

Low salt: low salt diet provided by the hospital kitchen for days 1-3.
High salt: free diet supplemented with 200 mmol sodium (20 Slow Sodium tablets taken twice daily) for days 4-6.
Free diet: free diet for days 7-9.

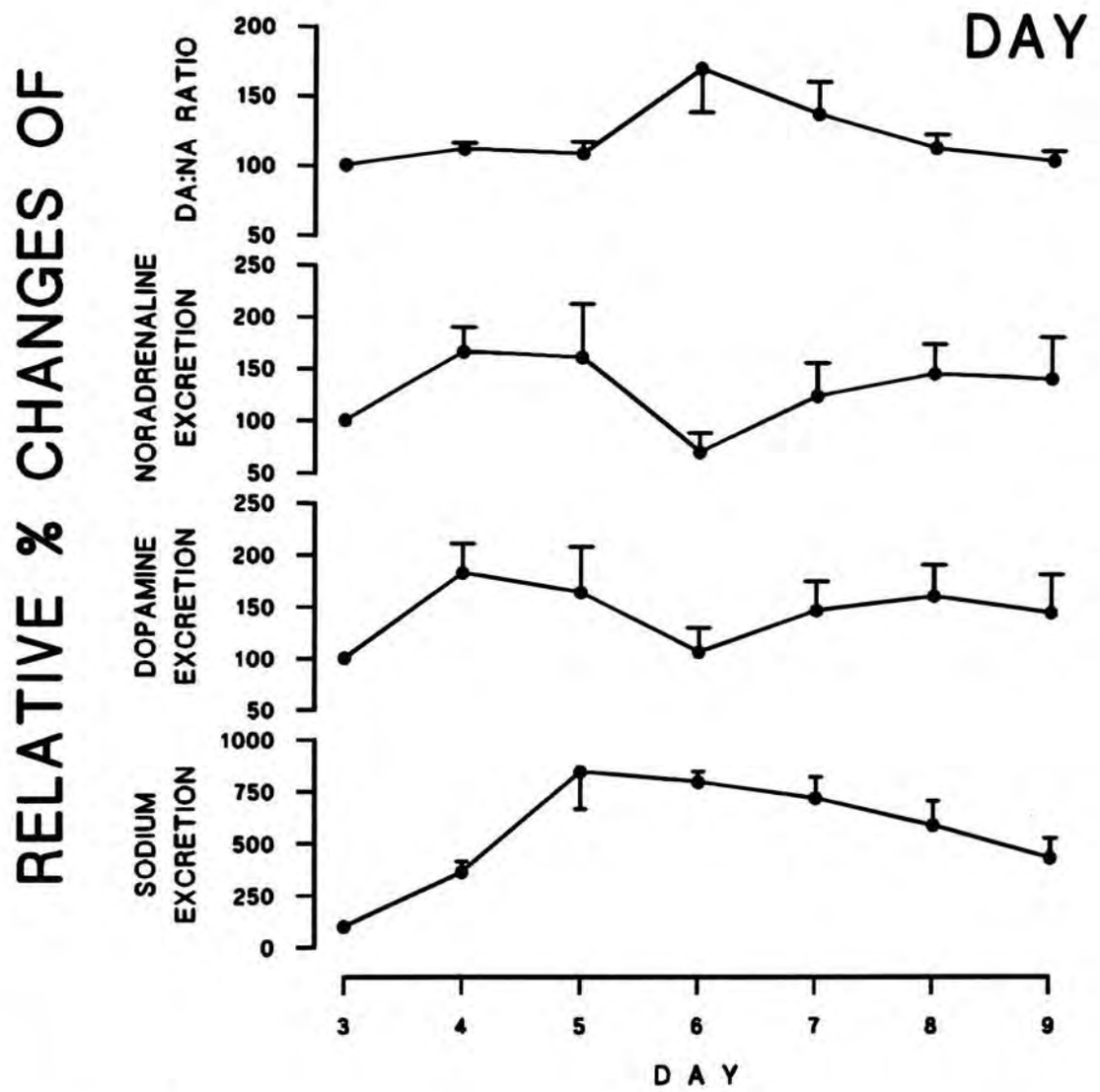


Figure 5-8: **Relative % changes w.r.t. end of low salt period for the excretion of sodium, DA, NA and DA/NA ratio during the day collection period (8 am to 10 pm).**

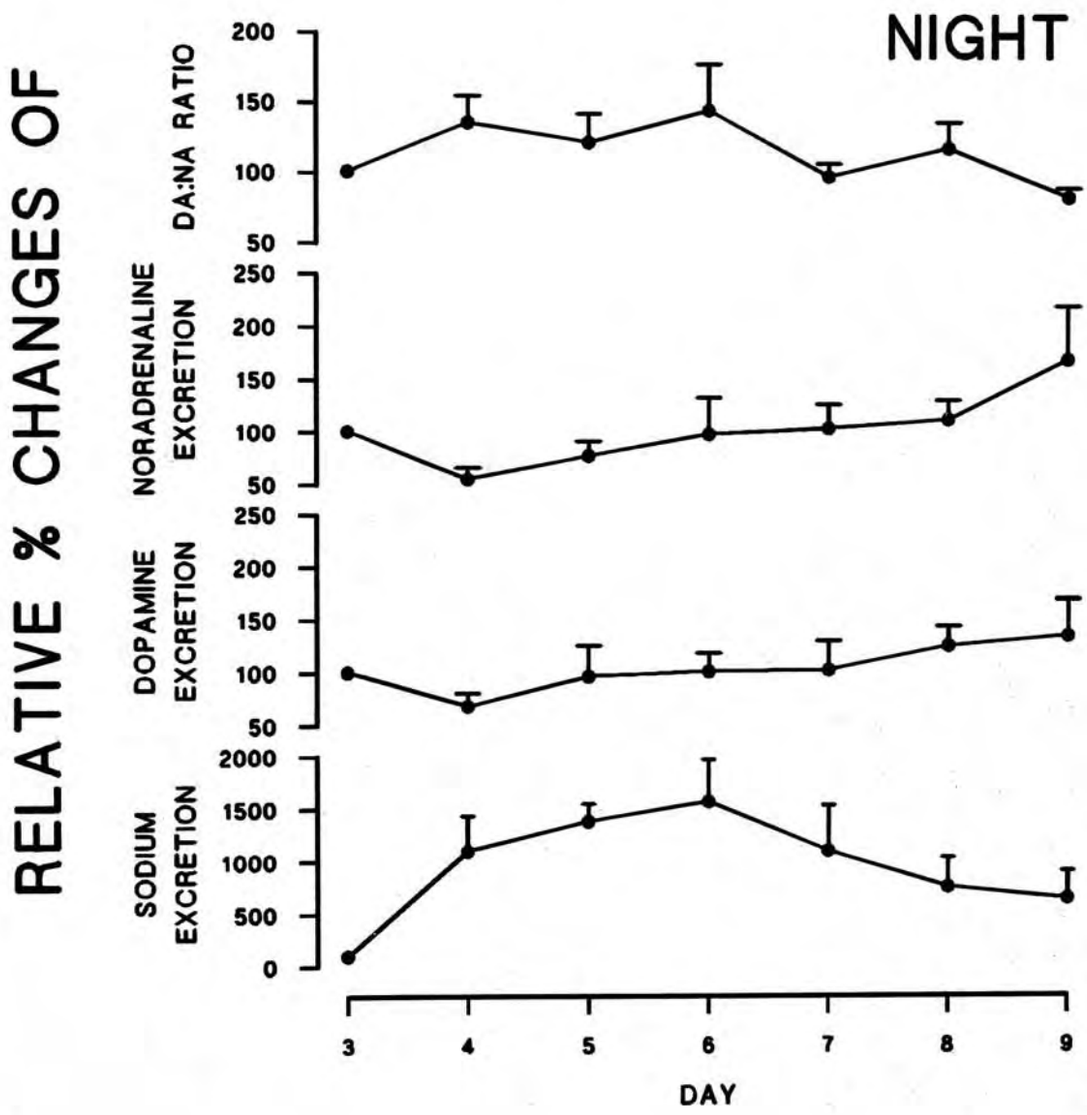


Figure 5-9: **Relative % changes w.r.t. end of low salt period for the excretion of sodium, DA, NA and DA/NA ratio during the night collection period (10 pm to 8 am)**

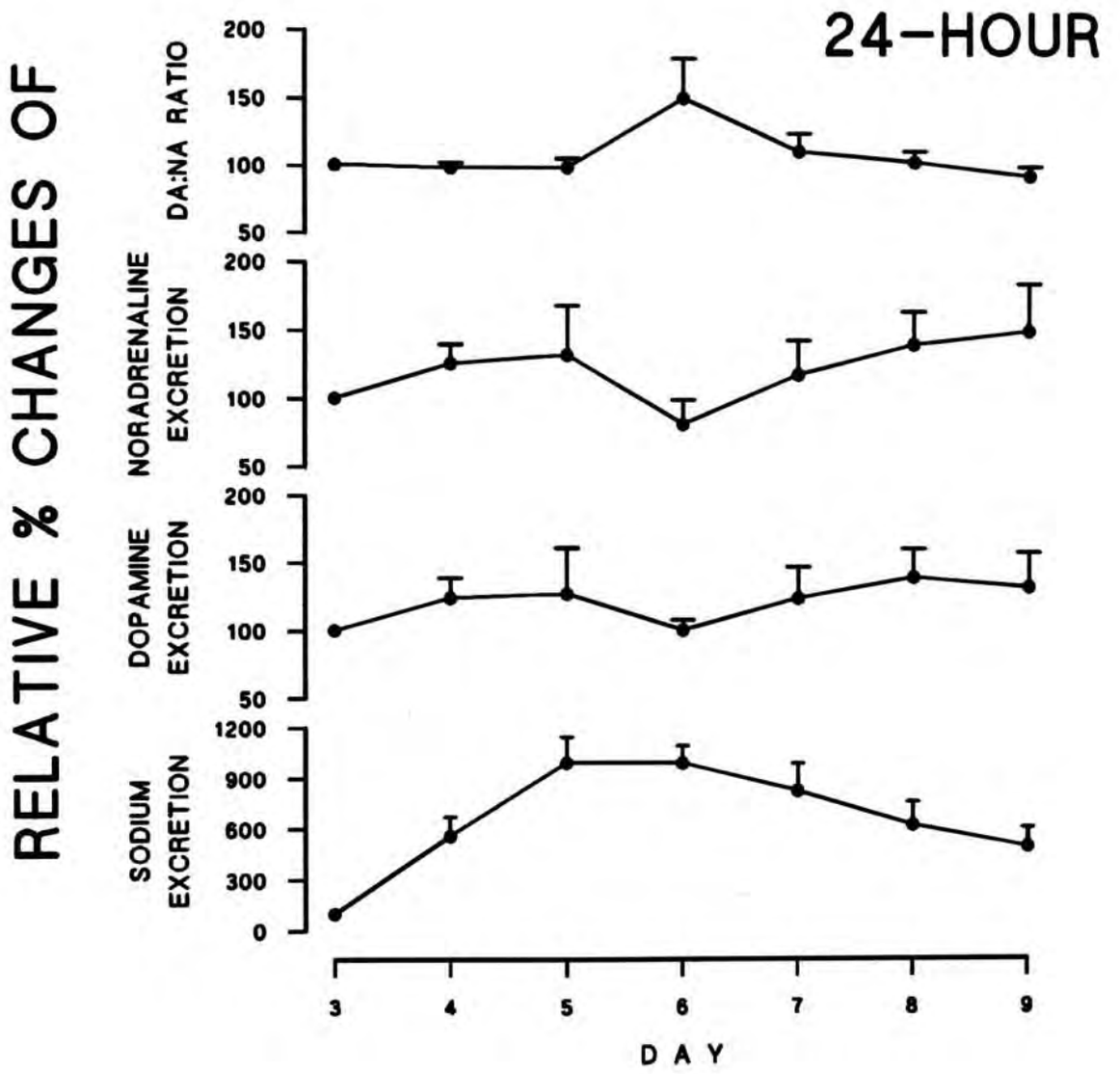


Figure 5-10: Relative % changes w.r.t. end of low salt period for the excretion of sodium, DA, NA and DA/NA ratio for 24 hour collection.

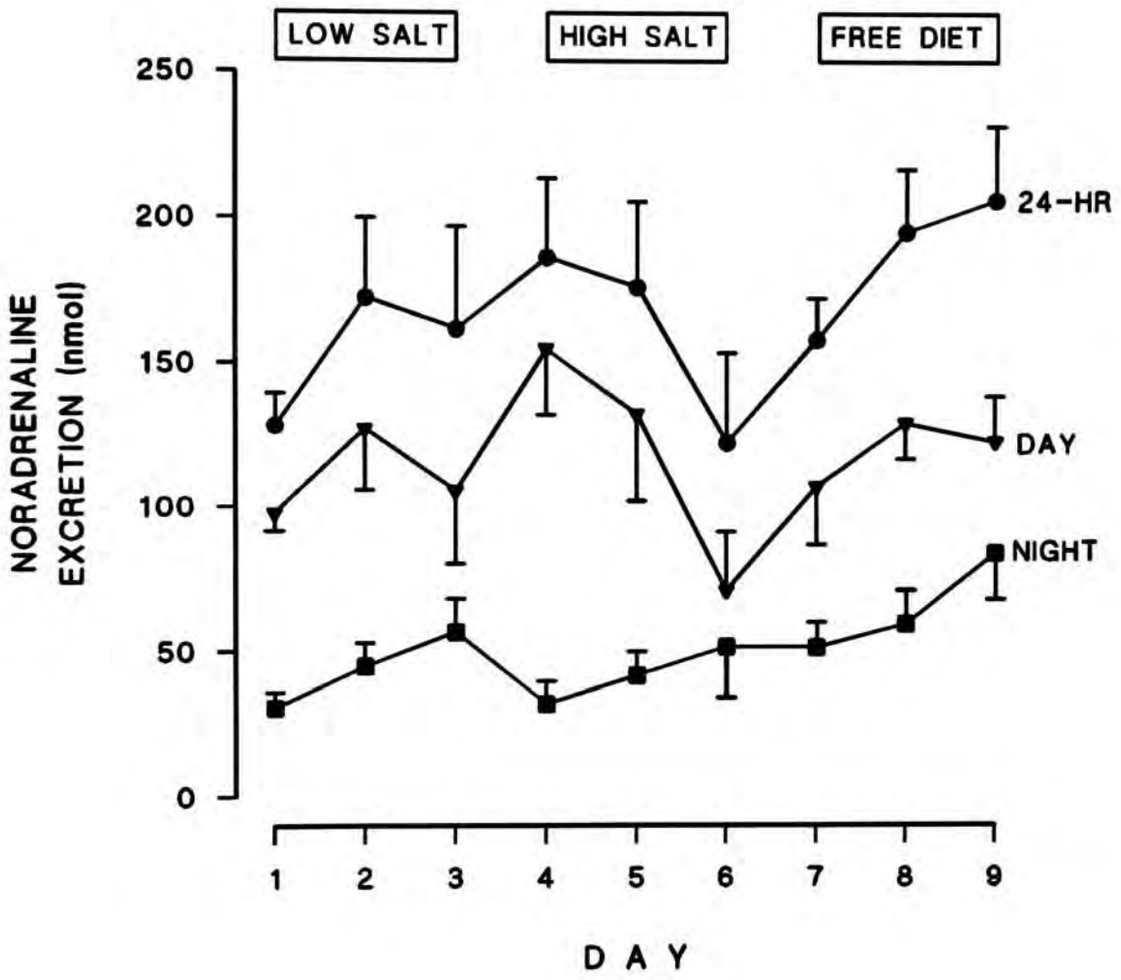


Figure 5-11: Excretion of NA during the low salt, high salt and free diet periods.

Low salt: low salt diet provided by the hospital kitchen for days 1-3.
High salt: free diet supplemented with 200 mmol sodium (20 Slow Sodium tablets taken twice daily) for days 4-6.
Free diet: free diet for days 7-9.

DA/NA Ratio

Figure 5-12 shows the DA/NA ratios for the day, night and 24-hour collections. For the 24-hour collection, the DA:NA ratios were relatively stable for days 1-5. It rose sharply on day 6 and then return to the previous level. ANOVA indicated that there were significant changes of the DA:NA ratios ($F=2.6468$, $p=0.0238$). However, the Scheffe test did not show significant difference of the ratio among the different days. A similar situation was also observed for the day collection ($F=11.867$, $p<0.0001$). On the other hand, DA:NA ratios for the night urine collections showed a different pattern and were higher than that of the day and 24-hour collections. The ratios were fluctuating throughout the study period and did not have a consistent pattern. Relative % changes of DA/NA ratios were similar to that show on Figure 5-12 except that the wide variation during the night collections was reduced (Figures 5-8, 5-9, 5-10).

Correlation between the different measured urine parameters

For the 9 day study period, the 24 hour sodium excretion was not correlated with the excretion of NA and DA, but correlated significantly with the DA:NA ratio ($r=0.300$, $p=0.0453$). However, this correlation disappeared when the data for day collections and night collections were studied separately. Furthermore, when the data were grouped into low salt, high salt and free diet periods, there was no correlation between sodium excretion and the excretion of CATS. There was a strong and positive significant correlation between the excretion of NA and DA throughout the study periods ($r=0.800$, $p<0.0001$). Such a relationship continued to hold true when the data were studied for the day and night collections, or separately for the 3 salt-loading periods.

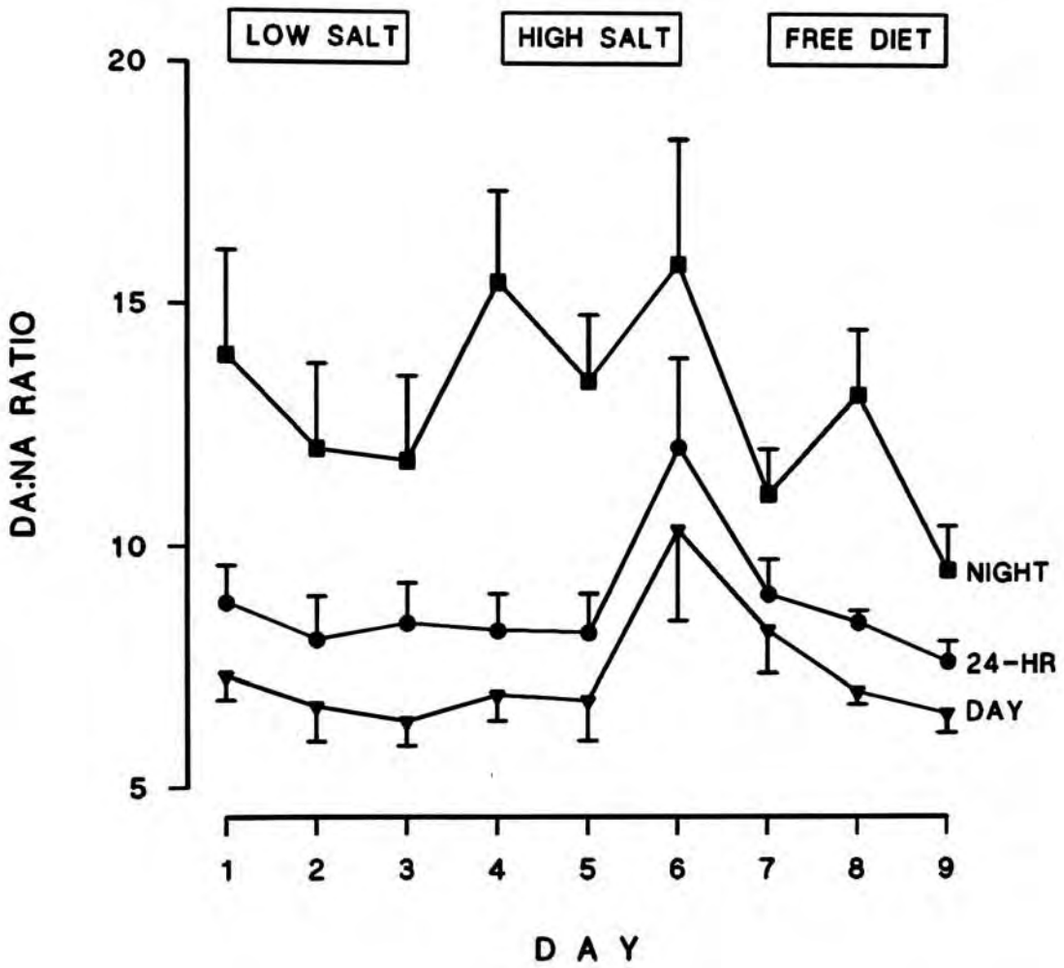


Figure 5-12: Urinary DA/NA ratio during the low salt, high salt and free diet periods.

Low salt: low salt diet provided by the hospital kitchen for days 1-3.
High salt: free diet supplemented with 200 mmol sodium (20 Slow Sodium tablets taken twice daily) for days 4-6.
Free diet: free diet for days 7-9.

DISCUSSION

Natriuresis following sodium loading is partly mediated by increased production of DA in the kidney which is reflected by an increase in urinary DA excretion. This has been demonstrated in man (Oates *et al* 1979) and animals (Ball *et al* 1978). It has been suggested that an impaired DA response to salt loading may lead to hypervolemia and hypertension in some individuals (Lee 1981). Urine DA failed to increase with salt loading in hypertensive patients (Harvey *et al* 1984) and this appeared to occur in salt-sensitive rather than salt-resistant hypertensives (Gill *et al* 1988). Subsequent cross-sectional studies in different ethnic groups have demonstrated that Caucasians and Thais show a strong positive correlation between 24-hour sodium and DA outputs whereas other groups such as Iranians and black West Africans do not (Critchley *et al* 1989). This could indicate that the ethnic groups lacking the sodium-DA relationship may not show a renal DA response to salt loading and thus be more prone to salt-sensitive hypertension or else have another predominant mechanism for dealing with increased salt intake. Cross-sectional studies in normotensive Chinese subjects without a family history of hypertension, reported in Chapter 4, have also shown a positive correlation between urinary sodium and DA. It was anticipated that normal Chinese subjects would respond to oral sodium with increase in urinary DA similar to Caucasians.

Another mechanism which appears to contribute to the natriuresis following sodium loading is a reduction in sympathetic activity which can be demonstrated by reduction in urinary NA excretion (Alexander *et al* 1974, Weinberger *et al* 1982, Castellano *et al* 1986, Gill *et al* 1988). Furthermore, plasma ESTI concentration has been reported to increase after oral salt loading in man (de Wardener *et al* 1981, Quintanilla *et al* 1988, Goto *et al* 1990) and in animals (Devynck *et al* 1987, Wauquier & Devynck 1989). The present study was set up to study the role of these 3 mechanisms in dealing with oral salt loading in healthy Chinese subjects.

In this study, the 5 subjects were first on a low salt diet of 40 mmol/d, then on a high salt diet of 390 mmol/d, and finally on their own free diets of 190 mmol/d (Figure 5-6). There was a 10-fold increase in sodium loading between the low salt and high salt periods. Significant ECF volume expansion was achieved as supported by a significant increase in body weight and decrease in plasma protein concentrations (Table 5-7). Since there was no increase in the MAP upon salt loading, these subjects can be considered as salt-resistant (Gill *et al* 1988).

The data show that 3 days of salt loading in this group of healthy Chinese subjects was not accompanied by any change in DA excretion (Figure 5-7). To minimize the effect of inter-individual variation, relative % changes of DA with respectto the end of the low salt diet were also calculated and analyzed. Again, there was no significant increase in DA excretion (Figures 5-8, 5-9, 5-10). Circadian variation of DA excretion has been reported (Kawano *et al* 1990). DA excretion is high during the day and low during the night. Thus, urine samples were collected for the day and night periods. DA excretion were higher for the day collection than that of the night collections throughout the 9 days (Figure 5-7). However, there was no significant increase of DA excretion in either the day or the night collections during high salt diet. These results are different from studies reporting an increase of 50-100% DA excretion in Caucasian subjects (Alexander *et al* 1974, Lee 1986, Gill *et al* 1988, Goldstein *et al* 1989). The lack of DA response was further supported by the absence of a significant correlation between sodium and DA excretion during the different salt loading periods. This is in contrast with results reported in Chapter 4 where normotensives without a family history of hypertension showed a significant correlation between urinary sodium and DA excretion. On the other hand, there have been animal studies showing that urine DA excretion does not increase during dietary salt loading. Urine DA excretion failed to increase in both Dahl salt-sensitive and salt-resistant rats (DeFeo *et al* 1987); spontaneous hypertensive rats and normotensive Wistar-Kyoto rats (Stier *et al* 1993) when given a 8% salt diet.

Plasma ESTI concentration was not increased after the salt loading in this group of Chinese subjects (Table 5-7). This result was different from that reported in the

literature (de Wardener *et al* 1981, Quintanilla *et al* 1988, Goto *et al* 1990). The discrepancy could be due to the use of different methods for measuring ESTI (Moreth *et al* 1987). Furthermore, the length of salt loading could be important for significant increase of ESTI. Chronic salt loading in rats showed that plasma ESTI continued to rise with the time of salt loading (Wauquier & Devynck 1989). In these studies, low salt and high salt periods were at least 5 days. A study on the kinetics of renal sodium excretion during changes in dietary sodium intake in man shows that it takes at least 4 days for the subjects to reach a new steady state for a low salt diet and 3 days for a high salt diet (Sagnella *et al* 1990).

Although there was no statistically significant reduction in NA excretion, there was a 30% lower NA excretion on the day of peak sodium excretion (Figure 5-11). Since this study was conducted with a small group of subjects, a larger number of subjects may be required to show statistical difference. It is possible that reduction in sympathetic nervous activity is the predominant mechanism for sodium excretion in Chinese subjects after dietary salt loading.

It has been suggested that the DA/NA ratio may be an important factor mediating sodium excretion due to the opposing effects of NA and DA on the renal handling of sodium. The previous experiment on water immersion in this Chapter showed that this ratio is important for sodium excretion. In this study, the DA/NA ratio was significantly different during the 9-day study period. This ratio was relatively stable for the day and 24-hour collections during both the low salt and free diet periods (Figure 5-12). It peaked on the last day of high salt diet (day 6) when sodium excretion was highest. However, the ratio did not show a consistent pattern for the night collections. Sodium excretion correlated weakly with this ratio for the 24-hour collection ($r=0.30$, $p<0.05$); but the correlation did not hold true when the analysis was performed for the day and night collections separately. However, the strong positive correlation between NA and DA observed is puzzling. If indeed, there were an opposite effects of NA and DA in renal handling of sodium in the Chinese, there should be an inverse relationship between them. The correlation was similar to the

findings in Chapter 4 and also a report for a group of young hypertensive Japanese (Saito *et al* 1994).

Dietary protein may be another factor influencing the results of this salt loading experiment. It has been reported that dietary protein has a significant effect on the excretion of DA (Williams *et al* 1986). Ingestion of 60 g of dietary protein induced a prompt increase of DA excretion by 50%. Subjects in this salt loading study were on their free diet after the low salt period. Variations in their protein intake can introduce variations in DA excretion. This may have increased the background 'noise' and the effect of salt loading was not clearly seen.

In this study of dietary salt loading with 5 Chinese subjects on free diet, a 10-fold increase in sodium intake did not show a significant increase in DA excretion nor plasma ESTI concentration. Although there was a reduction of NA excretion, it was not statistically significant. Extension of both the low salt and high salt periods could be necessary to elicit signals which are required to increase both DA and ESTI production. Furthermore, the subjects should be kept on a constant diet to reduce the influence of protein on the production of DA.

V. ORAL SALT LOADING UNDER CONTROLLED DIET

MATERIALS AND METHODS

The experimental design of this study was altered to improve on the previous study of oral salt loading while on a free diet (Section IV). To minimize the effects of other dietary factors on the excretion of DA, the diet in this study was controlled. A group of young male Chinese medical students was recruited for the study so that variations due to age could be reduced. Furthermore, both the low salt and high salt diet periods were extended to 5 days to ensure that the new steady state was attained.

Seven normotensive healthy male Chinese medical students (aged 19 to 22 years) were studied. These subjects were kept on a controlled hospital diet for 10-day study period (days -4 to 0 and days 1 to 5). The diet contained 1800 calories, 60 g protein, 20 mmol sodium and 60 mmol potassium. In addition, from days 1-5, subjects were given 20 "Slow Sodium" tablets (200 mmol), taken after breakfast. Subjects were asked to abstain from alcohol, medication, and strenuous exercise.

Blood pressure and body weight were measured on day 0 and day 6 as described in the previous section. Daily urine samples for the measurement of sodium and CATS were collected into 2 plastic bottles containing 100 ml of 0.5 M HCl. The day and night collections were similar to those described in the previous section. Before breakfast, venous fasting blood was collected daily from day -1 to day 6 for the measurement of sodium, protein, ESTI , ANP, renin and aldosterone. The methods of measurement and statistical analyses were as described in previous sections of this Chapter.

RESULTS

Urine sodium excretion

Figure 5-13 shows the sodium excretion for the day, night and 24-hour collections. ANOVA showed that there were significant increases in sodium excretion upon salt loading in all 3 collections ($F=56.20$, $p<0.0001$ for 24-hour collection; $F=40.24$, $p<0.0001$ for day collection; and $F=13.33$, $p<0.0001$ for night collection). From day -4 to day 0, sodium excretion decreased from 130 mmol/d to 32 mmol/d. Upon the supplementation with "Slow Sodium", sodium excretion increased 9 fold to 287 mmol/d. For the low salt period (days -4 to 0), excretion of sodium was higher during the day. With the high salt supplement, sodium excretion continued to increase for the first 3 days and then remained relatively constant for days 4 and 5 during the day. In contrast to the low salt period, sodium excretion during the night were higher than that during the day for the first 2 days. For days 3-5, sodium excretion decreased slightly and were lower than the day time.

Plasma results

Table 5-8 summarizes the effect of oral salt loading under controlled diet on body weight, MAP, plasma sodium, potassium, total protein and albumin. There were no significant changes in body weight and MAP. The mean plasma sodium increased significantly by 3 mmol/l after 5 days of oral salt loading. On the other hand, plasma potassium, total protein and albumin decreased upon salt loading significantly.

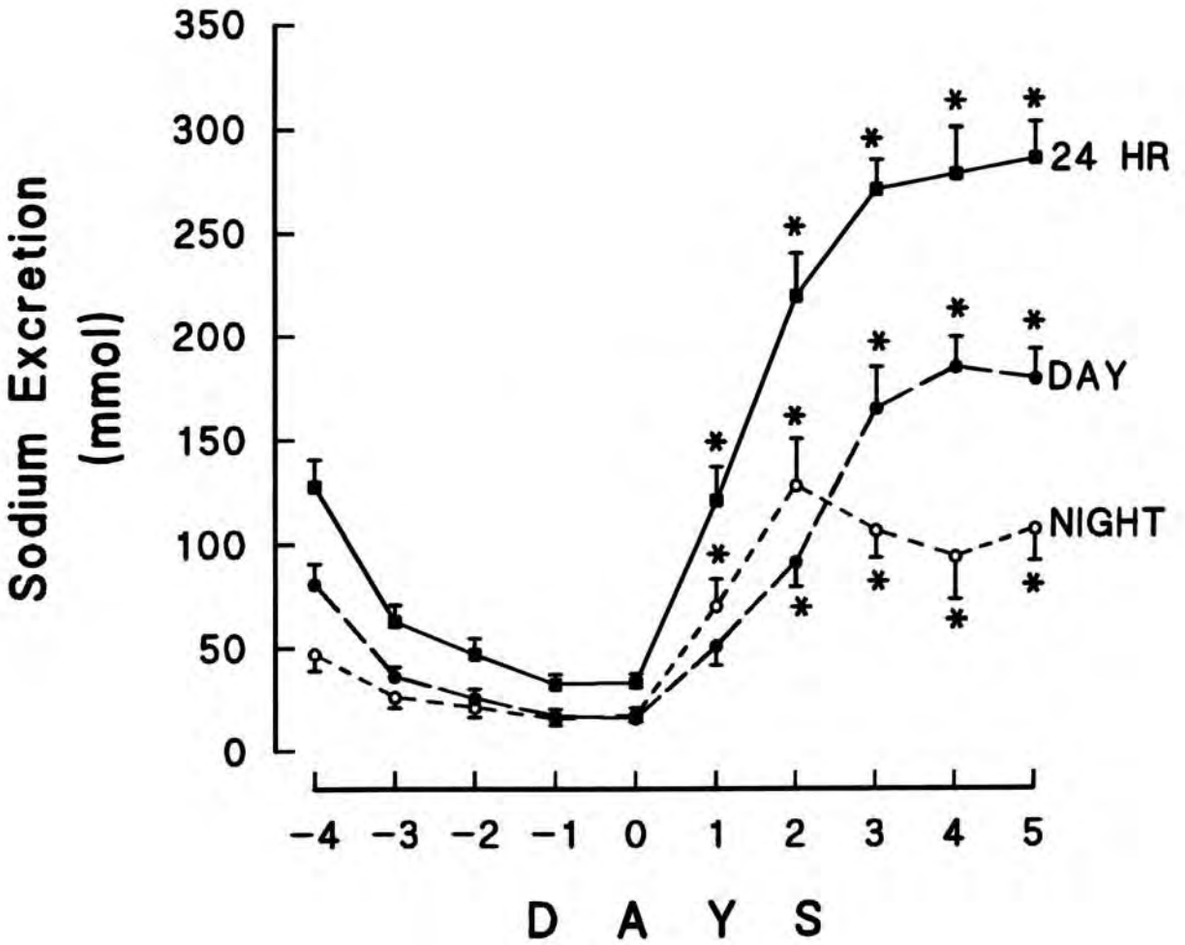


Figure 5-13: Excretion of sodium during the 10-day study period.
Days -4 to 0, on controlled low salt diet
Days 1 to 5, on controlled low salt diet + 200 mmol sodium supplementation.
* denotes significant different ($p<0.05$) on the day when compared to that on day 0 by Scheffe's test after ANOVA.

Table 5-8 **Effect of oral salt loading on blood pressure, body weight and other plasma variables.**

	Low salt diet (on day 0)	High salt diet (on day 6)
Body weight (Kg)	63.5 ± 5.87	63.7 ± 5.83
MAP (mmHg)	82.5 ± 5.51	80.6 ± 4.56
Plasma sodium (mmol/l)	137 ± 2.2	140 ± 1.4**
Plasma potassium (mmol/l)	4.2 ± 0.31	3.8 ± 0.23**
Plasma total protein (g/l)	86 ± 5.1	82 ± 3.6*
Plasma albumin (g/l)	50 ± 1.5	48 ± 2.5*

Results are given as mean ± SD
* denotes $p<0.05$ and ** denotes $p<0.02$ by Wilcoxon signed-rank test when compared with the low salt diet.

Figure 5-14 shows daily changes in some of the plasma natriuretic factors measured in this study. ANOVA showed significant changes of all 5 factors after 5 days of salt loading. Plasma renin and aldosterone increased during the low salt period and then decreased significantly upon salt loading ($F=5.788$, $p=0.0007$ for renin; $F=15.27$, $p<0.0001$ for aldosterone). Plasma ANP increased gradually after salt loading, peaked on day 4 and then returned to a concentration similar to days 2-3 ($F=3.131$, $p=0.022$). Na, K-ATPase inhibitor decreased on the low sodium diet. On supplementation with 200 mmol of sodium, it increased significantly on day 3, peaked on day 5 and remained high on day 6 ($F=23.90$, $p<0.0001$). Plasma DLI increased significantly on day 2 and then remained relatively stable for the remaining 4 days ($F = 10.81$, $p<0.0001$).

Urine DA excretion

Figure 5-15 shows the DA excretion for the day, night and 24-hour collections. There were no significant differences in DA excretion on any of the collections. 24-hour DA excretion decreased slightly during the low salt diet and increased during the last 3 days of salt loading. After 3 days of low salt diet, DA excretion decreased during the day, but remained relatively stable during the night. Upon the first day of salt loading, DA excretion increased promptly during the day. However, during the night, DA excretion decreased paradoxically despite of the high sodium excretion. DA excretion continued to increase during the day after day 2, but it remained lower during the night.

Figure 5-15 also shows the relative % change of DA excretion in relation to that on day 0 during oral salt loading. DA excretion increased during the day but did not reach statistical significance. During the night, DA excretion was lower than that of the day. Furthermore, night time DA excretion were all lower than that of the low salt period.

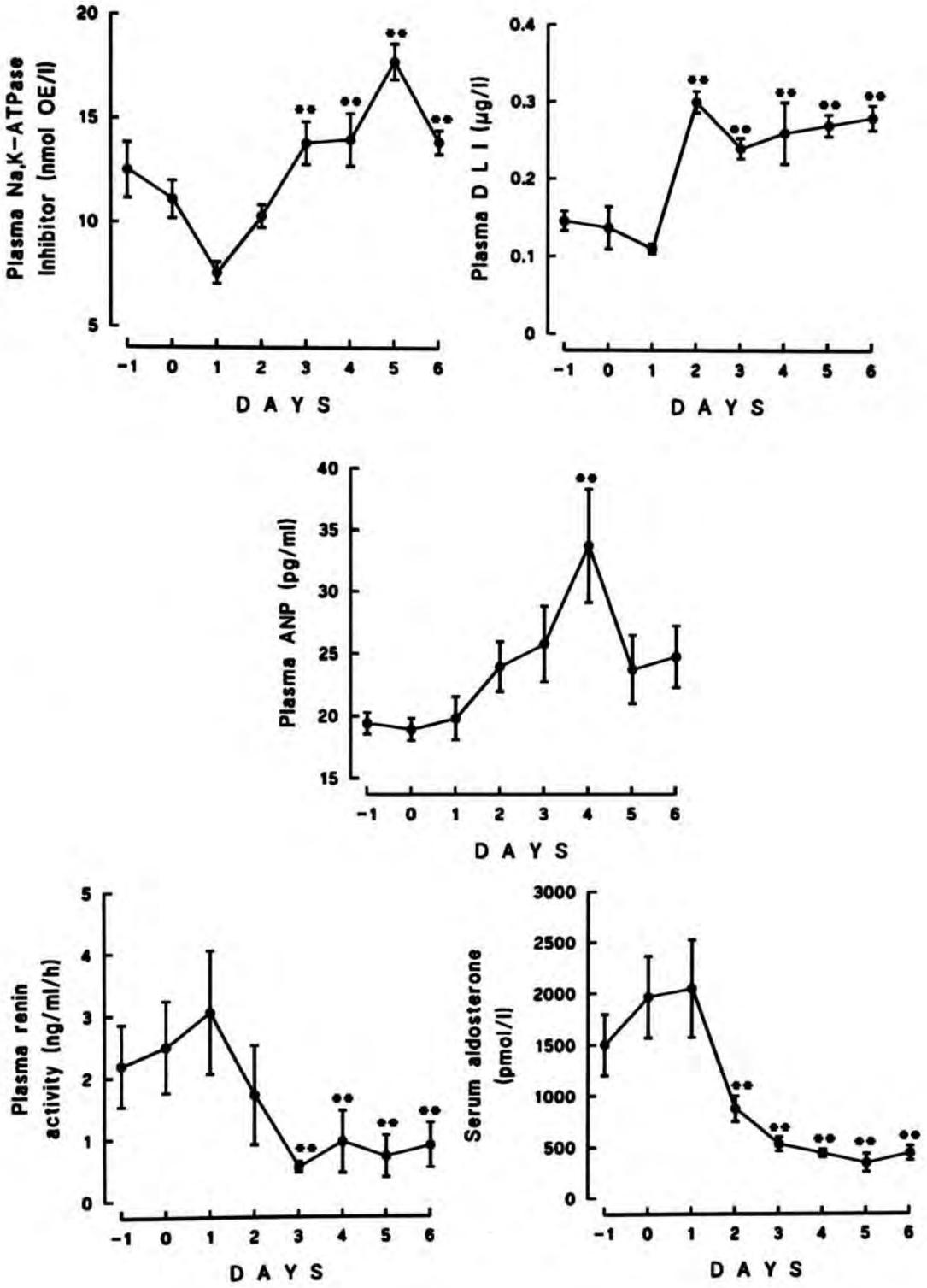


Figure 5-14: Effects of oral salt loading on different plasma natriuretic factors- Na, K-ATPase inhibitors, DLI, ANP, renin activity and aldosterone concentration.

** denotes that the plasma concentration/activity on the day was significantly different ($p<0.05$) from that on day 0 by Scheffe's test after ANOVA.

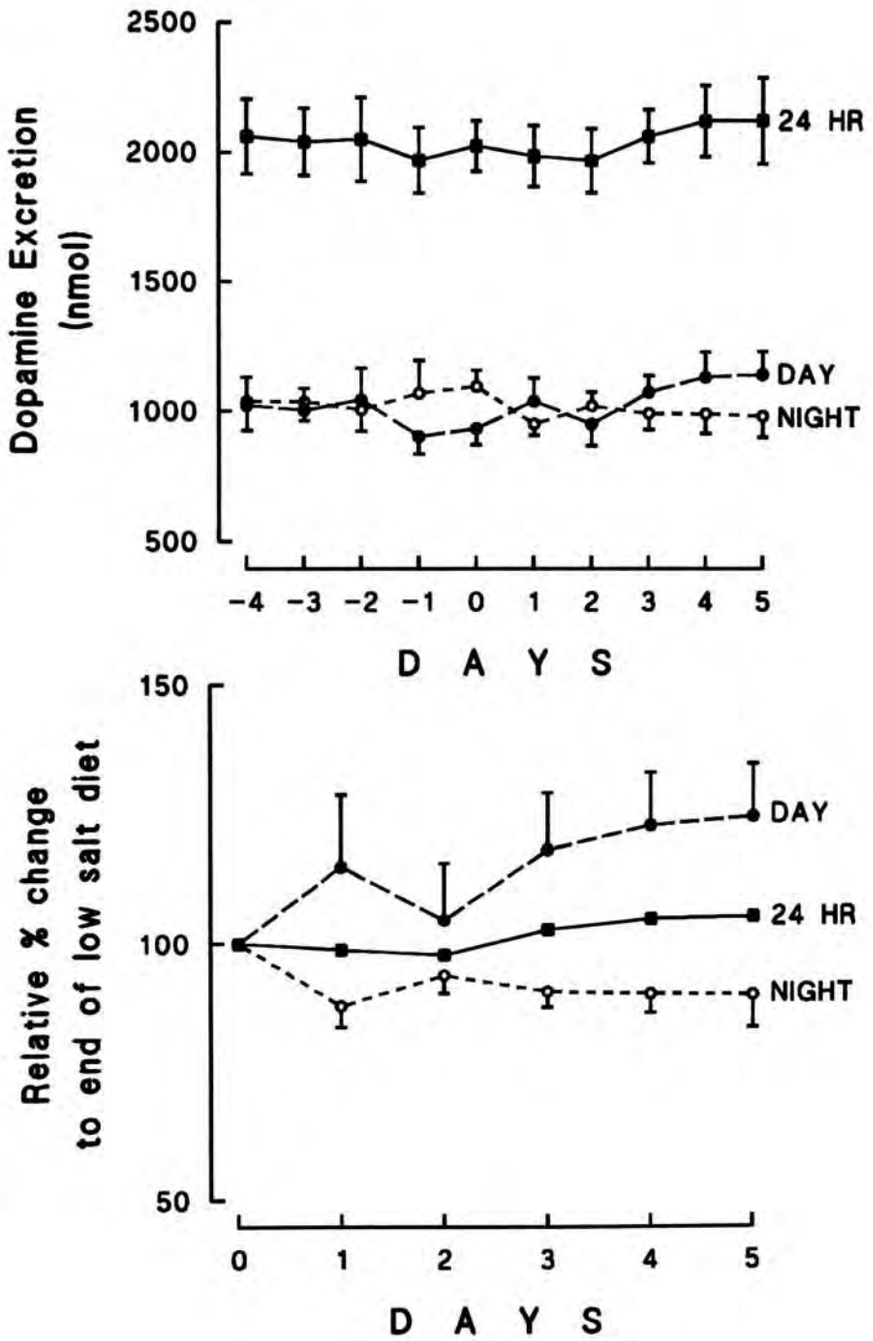


Figure 5-15: Excretion of DA during the 10-day study period.
(Top) DA excretion for the day, night & 24-hour collections
(Bottom) Percentage changes relative to day 0
Days -4 to 0, on controlled low salt diet
Days 1 to 5, on controlled low salt diet + 200 mmol sodium supplementation.

Urine NA excretion

Figure 5-16 shows NA excretion for the day, night and 24-hour collections. For the 24-hour collection, ANOVA showed that there were significant differences during the 10-day study period ($F=2.944$, $p=0.0065$). However, Scheffe's test failed to identify any pair of days that were significantly different. There were no significant differences between the day and night collections. NA excretion were higher during the day throughout the entire study period. With the 24-hour collection, NA excretion maintained an inverse relationship with sodium excretion. It increased during the low salt period and decreased for the first 3 days of the high salt period. On day 4, NA excretion increased and it decreased again on day 5. The increase on day 4 was mainly due to the increase of NA excretion during the night. NA excretion were relatively stable during the day during the last 2 days of study.

Figure 5-16 also shows the relative percentage changes in NA in relation to day 0. It shows that NA excretion decreased during the first 3 days of high salt period. However, NA excretion increased sharply during the night on day 4 and then returned to a lower level.

DA/NA ratio

Figure 5-17 shows the DA/NA ratios for the day, night and 24-hour collections. There were significant changes in the ratio for all 3 collections ($F=8.970$, $p<0.0001$ for the day collection; $F=3.552$, $p=0.0016$ for the night collection; $F=5.505$, $p<0.0001$ for 24-hour collection). DA/NA ratio paralleled the sodium excretion during the day. It decreased during the low salt period and increased significantly during the last 3 days of salt loading. DA/NA ratio was higher during the night throughout the study period. The ratio also showed larger variations during the night. It fluctuated between day -4 to day 0 and then showed a large increase from day 1 to 3. On day 4, the ratio decreased abruptly at night and increased again the next night. The relative percentage changes in relation to day 0 show that the ratios were all higher than day 0 after salt supplementation.

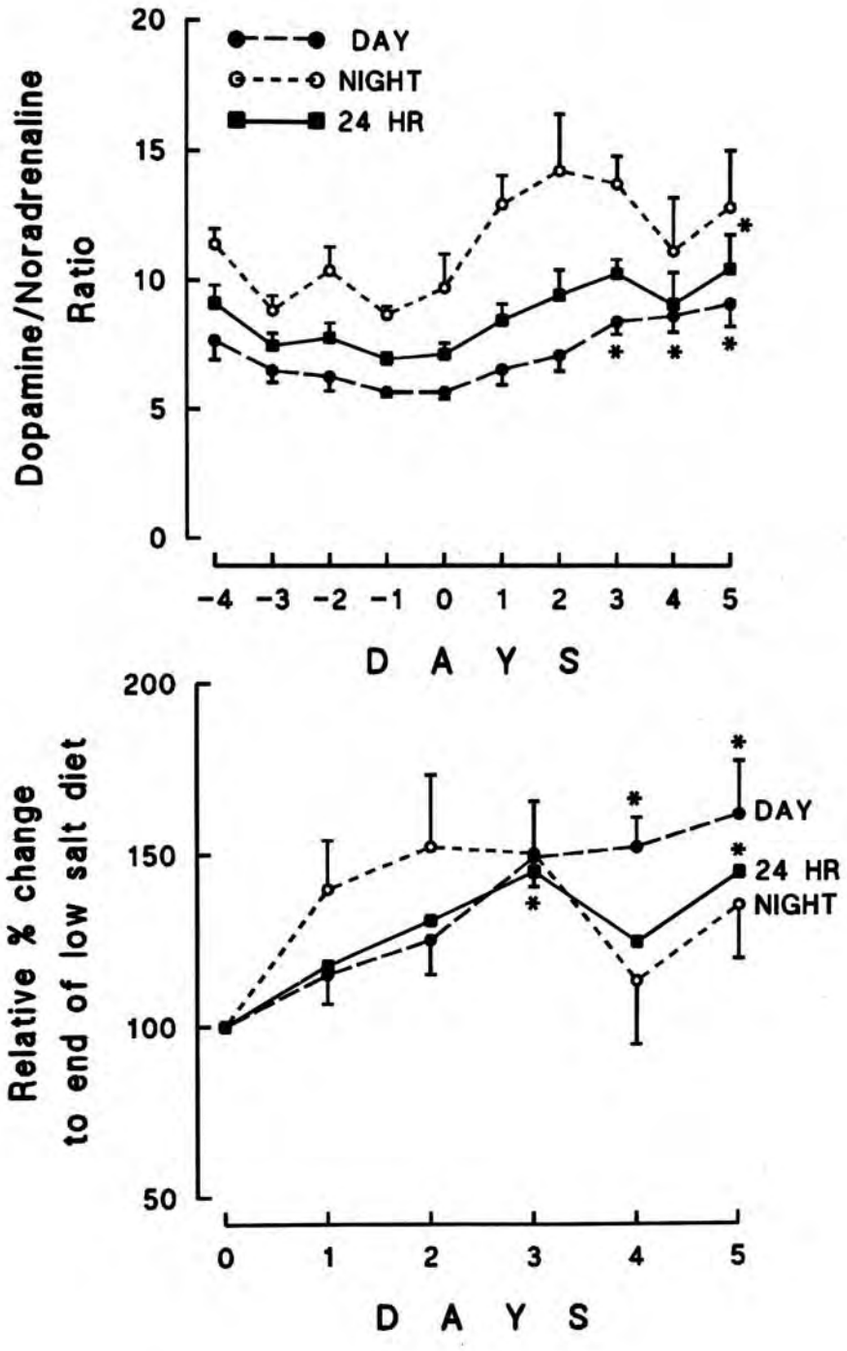


Figure 5-17: Changes of DA/NA ratio during the 10-day study period. (Top) DA/NA ratios for the day, night & 24-hour collections (Bottom) Percentage changes relative to day 0

Days -4 to 0, on controlled low salt diet

Days 1 to 5, on controlled low salt diet + 200 mmol sodium supplementation.

* denotes significant different ($p<0.05$) on the day when compared to that on day 0 by Scheffe's test after ANOVA.

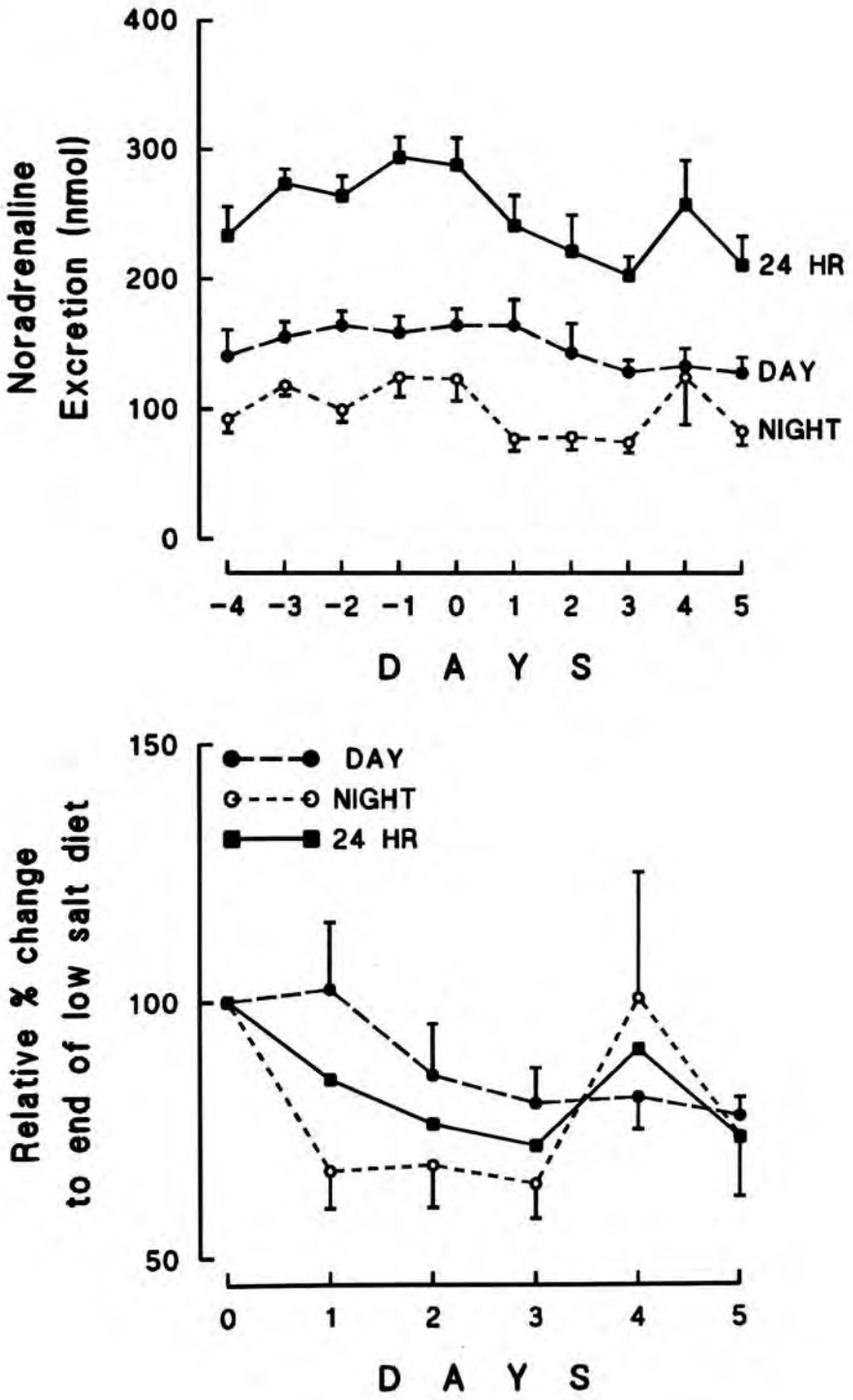


Figure 5-16: Excretion of NA during the 10-day study period.
(Top) NA excretion for the day, night & 24-hour collections
(Bottom) Percentage changes relative to day 0
Days -4 to 0, on controlled low salt diet
Days 1 to 5, on controlled low salt diet + 200 mmol sodium supplementation.

Correlation between the different measured variables

There were significant correlations between the different natriuretic factors during oral salt loading except for ANP. Plasma ANP showed no correlation with any of the other 4 factors measured in this study. Multiple linear regression showed that plasma sodium was significantly correlated with Na, K-ATPase inhibitor (β=0.7059, $p<0.0001$). When Na, K-ATPase inhibitor was used as a dependent variable, it correlated significantly with plasma sodium (β =0.6068, $p<0.0001$) and inversely with serum aldosterone (β=−0.276, p= 0.021). When DLI was used as a dependent variable, it only correlated inversely with aldosterone (β=−0.495, p=0.001).

Sodium excretion correlated significantly with the DA/NA ratio during day time (β =0.602, p=0.0001), but not during night time. Furthermore, the excretion of DA correlated significantly with NA excretion only for day collections (r=0.551, p=0.0006), not for the night collections.

DISCUSSION

The oral salt loading study described in the previous section failed to show any significant changes in urine DA excretion and plasma ESTI concentration. The present study was designed to improve on some of the possible experimental design faults that might have contributed to the lack of DA and ESTI response. For example, the duration of the low salt and high salt diets was extended to 5 days to ensure that a new steady state was reached (Sagnella *et al* 1990). The subjects were under the same controlled diets throughout the study period so that variations of DA and other natriuretic factors due to variation in other constituents in the diet, e.g. protein intake, can be minimized.

In this study, the 7 medical students were taking a low salt diet for the 10-day study period (day -4 to day 5). On day 1, they were given 200 mmol of sodium supplement as "Slow Sodium". There was a 9-fold increase in sodium excretion between the low salt and high salt periods (Figure 5-14). Suppression of plasma renin and aldosterone (Figure 5-13) shows that significant volume expansion was achieved. These students did not show a significant change in MAP during the high salt periods and therefore are salt-resistant.

Plasma samples were collected daily from day -1 to day 6 for the measurement of natriuretic factors. Fasting blood samples were taken early in the morning and before taking "Slow Sodium" tablets on days 1-5. The responses observed are the results of the previous days intake.

There was a significant increase in plasma ESTI concentration during the salt loading period (Figure 5-13). The Na, K-ATPase inhibitor increased significantly on day 3 whereas the increase of DLI was seen on day 2. The increase of plasma ESTI during oral salt loading is consistent with other studies (de Wardener *et al* 1981, Quintanilla *et al* 1988, Goto *et al* 1990). The amount of salt loading was comparable between the present study and the previous one described in section IV where there was no difference in plasma ESTI concentration between the low and high salt periods. This

suggests that the duration of low salt and high salt period could be critical to induce a significant response of plasma ESTI. Furthermore, there was a dissociation between plasma Na, K-ATPase inhibitor and DLI.

A significant increase in plasma ANP was observed on day 4 (Figure 5-13). It increased from 18.9 pg/ml on day 0 to 33.7 pg/ml on day 4. Increase in ANP during oral salt loading has been reported (Sagnella *et al* 1987, Solomon *et al* 1987, Sagnella *et al* 1989, Doorenbos *et al* 1990). The magnitude of increase was closely associated with cumulative sodium balance (Sagnella *et al* 1989). In this present study, the increase in ANP was nearly 2-fold with a 9-fold increase in sodium excretion. This increase was similar to that reported in the literature. However, plasma ANP decreased abruptly on day 5. Again, reference to such a phenomenon could not be found. The decrease in plasma ANP could be a contributing factor for the surge in plasma Na, K-ATPase inhibitor. However, the reason for this decrease in ANP cannot be easily explained on the present data. Daily changes in plasma ANP are not usually reported in the literature. Sodium excretion reached a new steady state on day 3 (Figure 5-14, Sagnella *et al* 1990).

Despite increasing the duration of salt loading to 5 days and controlling the diet throughout the study, there was still no significant change in urinary DA excretion during the day or night (Figure 5-15 top). In previous studies of DA response to oral salt loading, 24-hour urinary excretion has been examined. Under the present experimental design, separate collections of day and night urine samples revealed that DA excretion correlated with sodium excretion better during the day than the night. The circadian variation of DA excretion reported (Kawano *et al* 1990) was not observed during the low salt period. Upon salt loading, there was a 23% increase in day time DA excretion from day 0 to day 5. However, this increase did not reach statistical significance. During the same period of time, there was a 10% decrease in night time DA excretion. When the relative percentage change in DA excretion (Figure 5-15 bottom) was examined it confirmed that night time DA excretion was lower than day 0 during salt loading. Such a phenomenon has not been reported previously. From day 1 onwards, there was an interesting pattern in DA excretion.

When there was a higher increase in DA excretion during the day, there was a corresponding lower DA excretion during the night.

There was no statistical significant change in NA excretion during the 10-day study period for any of the 3 collections (Figure 5-16 top). NA excretion correlated inversely with the sodium excretion. During the low salt period, NA excretion increased gradually. On day 1 of oral salt loading, NA excretion decreased for 3 days. Then there was a sharp rise in NA excretion during the night. Increase in NA excretion could be due to the activation of the sympathetic nervous system or the removal of inhibitory actions on the nervous system. This sudden increase could be in response to the lowering of plasma ANP. It has been reported that ANP can inhibit the sympathetic nervous system (Goetz 1990). Thus, the inhibitory effects of ANP on the sympathetic system may be a signal for the increase in production of ESTI.

DA/NA ratio correlated positively with the sodium excretion. The ratio decreased during the low salt period and increased during the sodium supplementation. There were significant changes of DA/NA ratio for the day and 24-hour urine collections (Figure 5-17), but not for the night collection. With multiple regression analysis, sodium excretion correlated significantly with this ratio during the high salt period during the day, but not during the night. Furthermore, there was a significant correlation between DA and NA again during the day, but not during the night. The results were similar to the findings in Chapter 4, and also salt loading study on a free diet (Section IV).

In this study of oral salt loading under controlled diet in Chinese subjects, a 9-fold increase in sodium intake did not have significant increase in DA excretion. The urine DA/NA ratio correlated with the sodium excretion during the day, but not the night time.

CHAPTER 6

STUDIES ON THE EFFECTS OF SALT LOADING IN THE RAT

I. INTRODUCTION

Effects of salt loading on the urinary excretion of DA and ESTI have been commonly studied using the rat model. It has been shown that urinary excretion of DA increases during salt loading in the rat (Ball *et al* 1978). Furthermore, increased circulating ESTI has been shown in the rat while on a high salt diet (Wauquier & Devyneck 1989). However, their temporal relationship in natriuresis has not been studied. In the first study of this Chapter, the relative contributions and temporal relationship of urinary DA and ESTI in natriuresis were studied.

It has been suggested that in hypertensives, renal DA mobilization is impaired and ESTI increases to maintain salt balance (Lee 1987). In the second study of this Chapter, production of renal DA was inhibited by the use of carbidopa. Carbidopa inhibits dopa decarboxylase activity which is responsible for the conversion of L-dopa to DA in the renal tubules. Excretion of ESTI would be increased if Lee's hypothesis is correct.

II. TEMPORAL RELATIONSHIP BETWEEN EXCRETIONS OF DA AND ESTI DURING SALT LOADING IN THE RAT

MATERIALS AND METHODS

Six female Sprague-Dawley rats weighing 250 g were studied. They were fed ad libitum throughout the study with normal rat chow containing 0.2% sodium chloride (NaCl). The animals were placed in individual metabolic cages and given tap water to drink for 4 days. 24 hr urine samples were collected for two days (days -2 and -1). For days 1-7, the tap water was changed to 18 g/l NaCl solution and urine collection was continued. Urine samples were collected in 6.0 M HCl and after recording the volume, they were stored at -70 °C.

Urinary sodium and creatinine concentrations were measured as described in Chapter 4. Urinary DA was measured as described in Chapter 3 and urinary ESTI was measured as described in Chapter 2. Excretion of sodium, DA and ESTI were related to creatinine in order to compensate for any loss of urine during collection and for any differences in lean body mass during the study.

For statistical analysis, the mean values of day -1 and -2 were used to represent the excretion before salt loading. The data were subjected to ANOVA and when the null hypothesis was rejected, Scheffe's comparisons were performed on the means. $p<0.05$ was considered significant.

RESULTS

Table 6-1 summaries the results. Urine sodium excretion on day 1 was significantly higher than pre-salt loading . Sodium excretion reached a peak on day 2 and thereafter remained relatively stable. Urine DA excretion on day 1 was 9% higher than basal value but the difference was not significant. On day 2, the urinary DA excretion was 21% higher than the mean before the salt loading ($p<0.01$). Urine DA excretion reached a peak on day4 and remained high thereafter. On the other hand, urine excretion of ESTI on day 1 was 4.2 fold higher than the basal values. Figure 6-1 shows the relative changes of the measured parameters with time. The excretion of ESTI paralleled the rise in sodium whereas DA excretion lagged behind.

Table 6-1: Effect of salt loading on the excretion of sodium, DA and ESTI in rats.

Day	Urine volume (ml/d)	Sodium / Cr (mmol/mmol Cr)	DA / Cr (nmol/ mmol Cr)	ESTI / Cr (nmol OE/mmol Cr)
-2	10.0 ± 1.9	37 ± 3.3	240 ± 22.0	0.46 ± 0.15
-1	9.8 ± 3.0	39 ± 5.5	211 ± 21.3	0.40 ± 0.15
1	27.5 ± 8.3*	174 ± 48.0**	246 ± 34.6	1.80 ± 0.30**
2	29.2 ± 11.2*	210 ± 46.4**	273 ± 31.9*	2.67 ± 0.45**
3	24.7 ± 10.6*	203 ± 33.5**	329 ± 45.7**	2.61 ± 0.44**
4	23.3 ± 7.0*	192 ± 29.0**	336 ± 56.1**	2.83 ± 1.10**
5	17.1 ± 5.9*	190 ± 13.8**	308 ± 53.2**	3.74 ± 1.07**
6	27.6 ± 8.3*	186 ± 17.6**	305 ± 39.0**	2.92 ± 0.96**
7	19.2 ± 5.8*	206 ± 18.2**	371 ± 46.7**	2.76 ± 0.64**

Results are mean ± SD, n=6
* for $p<0.01$, ** for $p<0.001$ significantly different from the mean of days -1 and -2

Sodium / Cr denotes for sodium / creatinine ratio
OE for ouabain equivalent

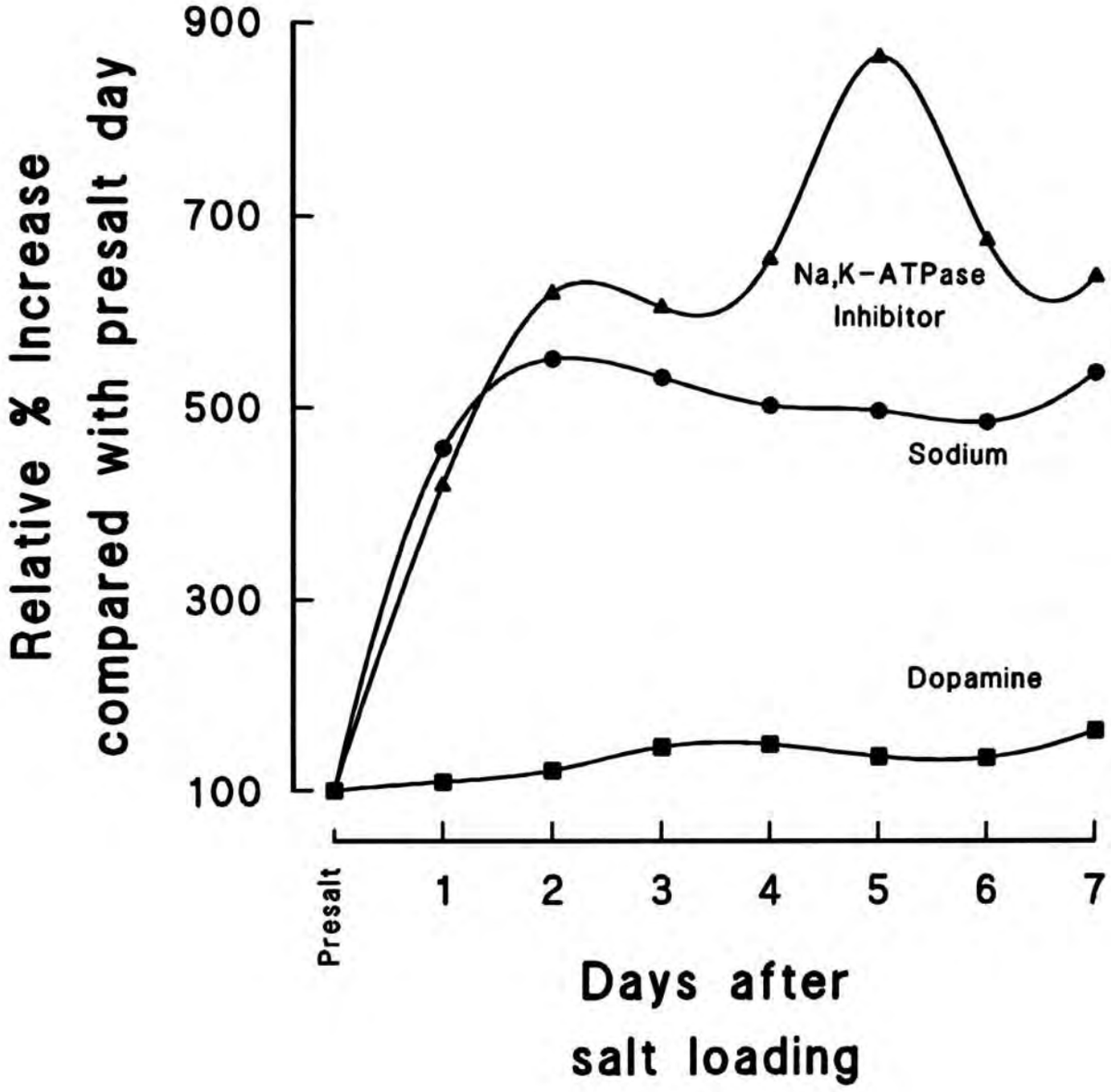


Figure 6-1: **Relative changes of excretions of sodium, DA and ESTI during salt loading in rats.**

DISCUSSION

High salt intake has been shown to cause an increase in excretion of DA (Ball *et al* 1978, Oates *et al* 1979) and ESTI (Wauquier & Devyneck 1989). The evidence for the release of ESTI during salt loading comes from direct measurement of the inhibitory activity (Poston *et al* 1982, Wauquier & Devyneck 1989) or from indirect measurement of Na,K-ATPase activity of circulating cells (Quintanilla *et al* 1988). This experiment has been set up to investigate the relative importance of DA and ESTI to the natriuresis of salt loading in the rat by examining the temporal relationships. The results confirm previous observations that high salt intake causes an increase in DA excretion (Ball *et al* 1978). The magnitude of increase (approximately 50% on day 4) is similar to that reported by others (Alexander *et al* 1974, Ball *et al* 1978, Oates *et al* 1979).

The results also confirm that high salt diet causes an increase in ESTI (Gonick *et al* 1977, Poston *et al* 1982, Wauquier & Devyneck 1989). So far there has been no reports on the temporal relationship between DA and ESTI during salt loading. Results of this study show that ESTI closely followed the excretion of sodium (Table 6-1) where as the excretion of DA lagged behind by a day. Furthermore, unless the natriuretic effect of DA is several fold higher than other natriuretic factors, the observed increase in DA excretion may not be quantitatively adequate to explain the natriuresis (Figure 6-1). However, it is possible that the local concentration of DA at the tubule may be high enough to account for this degree of natriuresis.

These observations on DA could be interpreted in two ways. Firstly, DA is not important in the natriuresis of salt loading and the increased excretion of DA is secondary to the excretion of sodium. Observations such as lack of increase in DA excretion during headout water immerison as described in Chapter 5 and the lack of effect of carbidopa on the natriuresis induced by saline infusion (Jeffrey *et al* 1989) support this theory. However, there are other observations which support a natriuretic role for DA. For instance, administration of carbidopa to healthy subjects reduced sodium excretion suggesting that DA has a tonic role in the excretion of sodium (Ball *et al* 1971, Lee 1987). Dopaminergic blockade by the use of the DA

receptor antagonist metoclopramide abolished the natriuretic response to saline infusion (Krishna *et al* 1985). Secondly, DA may be important in maintaining the natriuresis initiated by other factors such as ESTI or ANP.

There was a surge in the excretion of Na, K-ATPase inhibitor on day 5 of salt loading (Figure 6-1). However, the surge only lasted for one day and the excretion decreased again to the level similar to that of day 4. The phenomenon cannot yet be explained. It may be due to the reduced activities of some natriuretic factors after 4 days of salt loading, for example ANP. If so, the excretion of ESTI would have to increase to maintain sodium excretion. This surge could signal the activation of some other natriuretic factors. As suggested by de Wardener's hypothesis, the action of the Na, K-ATPase inhibitor is non-specific, and a very high concentration could be unfavourable to the system leading to increase in blood pressure.

The present results strongly suggest a role for ESTI in the natriuresis of salt loading and that the increase in ESTI precedes that of DA.

III. ROLES OF DA AND ESTI IN NATRIURESIS IN RATS TREATED WITH CARBIDOPA

MATERIALS AND METHOD

Twenty-four male Sprague-Dawley rats of 1 month old were studied. They were fed ad libitum throughout the study with normal rat chow containing 0.2% sodium chloride. The animals were placed in individual metabolic cages and given tap water to drink for 3 days. They were then randomly divided into 4 groups: Group A - low salt group with tap water to drink; Group B - high salt group with 18 g/l of NaCl solution to drink; Group C - low salt + carbidopa; and Group D - high salt + carbidopa. Carbidopa (kindly supplied by Merck, Sharp & Dohme, PA, USA) was dissolved in water and fed orally to Groups C and D twice daily at a dose of 120 mg/Kg body weight/day. Groups A and B were also fed orally with water twice daily at the time of feeding the 2 experimental groups.

24 hour urine samples were collected into 0.5 M HCl solutions for 7 days. Groups A and C had a smaller urine volume and the containers were filled with 4 ml of acid solution; while Groups B and D had larger urine volumes and the containers were filled with 10 ml of acid. Measurement of DA was performed on the day after the completion of urine collection to avoid any artifacts introduced due to the instability of CATS in the urine samples. Aliquots of the urine were stored at -70 °C for the measurement of urinary ESTI. Methods of measuring sodium, creatinine, DA and ESTI were as described in section II of this Chapter.

Excretions of sodium, DA and ESTI were also related to creatinine in order to compensate for any loss of urine during collection and for any differences in lean body mass during the study. For statistical analysis, one-way ANOVA was used to compare results between the 4 Groups and also for changes of the excretion within the Group. Significance was to set to $p<0.05$.

RESULTS

Figure 6-2 shows the excretions of sodium in the 4 groups of rats over the study period. Within each group of rats, there was no significant difference of sodium excretion over the 7 days. Sodium excretions were almost 10-fold higher in the high salt groups than the low salt groups. Sodium excretion was higher in the carbidopa treated high salt rats (Group D) compared to the high salt one (Group C), but the difference did not reach statistical significance. Thus, inhibition of DA production by carbidopa treatment had no effect on sodium excretion as there was no significant difference between the control and the treatment groups for both low salt and high salt rats.

Figure 6-3 shows the excretion of DA in the 4 groups of rats over the study period. DA excretion in Group A (low salt) decreased over the 7 days ($F=2.34$, $p=0.053$). DA excretion in the Group B (high salt), though not statistically significant, rose from day 1, peaked on day 5, and then decreased on the last 2 days. On the other hand, DA excretion in Group D (high salt rats treated with carbidopa) increased significantly over the 7 days ($F=2.618$, $p=0.035$). There was also a slight by not significant increase of DA excretion in Group C (low salt rats treated with carbidopa). Both high salt groups (Groups B&D) had higher DA excretions than the corresponding control groups (Groups A&C). Group B (without carbidopa treatment) showed a significantly higher DA excretion than the corresponding low salt group (Group A) after the second day of salt loading. However, the carbidopa treated high salt rats (Group D) did not have significantly higher DA excretion than Group C. After 4 days of carbidopa treatment, the high salt rats began to show an increase in DA production on day 5 and onwards.

Figure 6-4 shows the excretions of Na, K-ATPase inhibitor in the 4 groups of rats over the study period. There were significant changes in the excretion of inhibitor within each group over the 7 days ($p<0.001$ for each group). In groups that did not receive carbidopa treatment (Groups A&B), excretion of the inhibitor peaked on day 5 and then returned to a level even lower than day 1. The high salt rats (Group B) had significantly higher inhibitor excretion than that of the low salt rats (Group A)

($F=7.99$, $p<0.0001$). For rats treated with carbidopa (Groups C&D), excretion of inhibitor was significantly higher than that of Group A, but not different from Group B. The pattern of excretion was also similar to that of Group B except that the peak was on day 4. Group D (high salt) tended to have higher excretion of inhibitor than that of the corresponding low salt group (Group C) but the difference did not reach statistical significance.

There were no significant difference in the body weights between the 4 groups of rats ($F=0.715$, $p=0.544$). Groups A, B and C all had significant increase in body weights during the study period, ranging from 34.7% to 47.9%. However, in Group D, the increase in body weight (24.3%) did not reach statistical signifiance.

Correlation studies were performed in the 4 groups of rats. There was a strong positive correlation between the excretion of sodium and DA in the high salt rats ($r=0.757$, $p<0.0001$ for Group B; $r=0.685$, $p<0.0001$ for Group D). There was a weak and inverse correlation between sodium and DA excretion in Group C ($r = -0.330$, $p=0.033$). In Group D, there was also a significant inverse correlation between the excretion DA and inhibitor ($r= -0.376$, $p=0.019$). Since there were inter-relationships between the 3 measured parameters in Group D, multiple regression analysis was performed. When sodium excretion was used as the dependent variable, it correlated significantly with DA excretion ($\beta =0.781$, $p<0.0001$) and inhibitor excretion ($\beta =0.254$, $p=0.048$). Furthermore, 47% of sodium excretion can be attributed to DA excretion and only 6% to inhibitor. When DA excretion was used as the dependent variable, it correlated significantly with sodium excretion ($\beta = 0.672$, $p<0.0001$) and inversely with inhibitor excretion ($\beta = - 0.349$, $p=0.0023$). Similarly, 47% of DA excretion can be attributed to sodium excretion, and 13% to inhibitor.

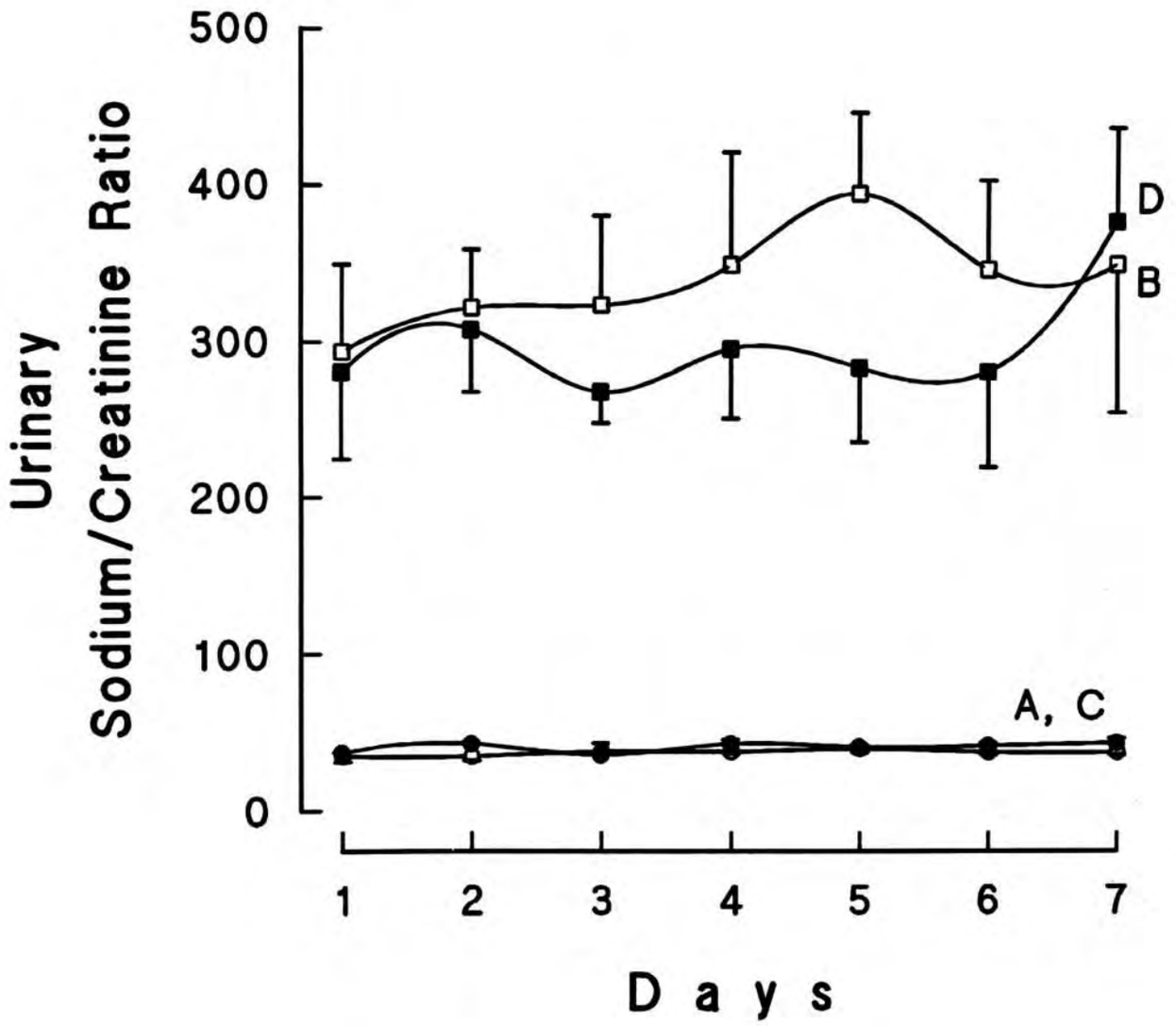


Figure 6-2: **Changes of sodium/creatinine ratio in the 4 groups of rats.**

Group A: low salt
Group B: high salt
Group C: low salt + carbidopa
Group D: high salt + carbidopa
Results as mean ± SEM

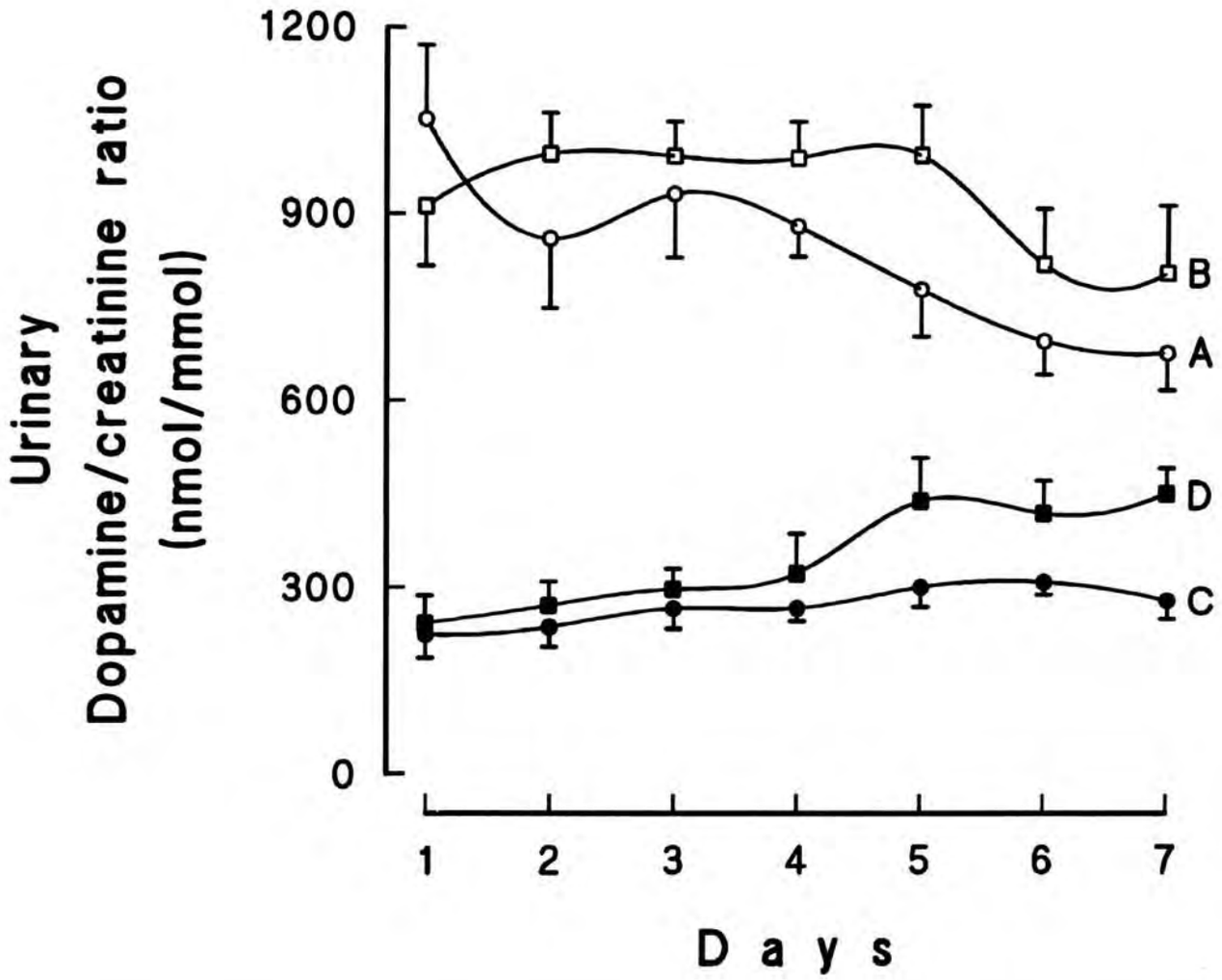


Figure 6-3: Changes of DA/creatinine ratio in the 4 groups of rats.

Group A: low salt
Group B: high salt
Group C: low salt + carbidopa
Group D: high salt + carbidopa
Results as mean ± SEM

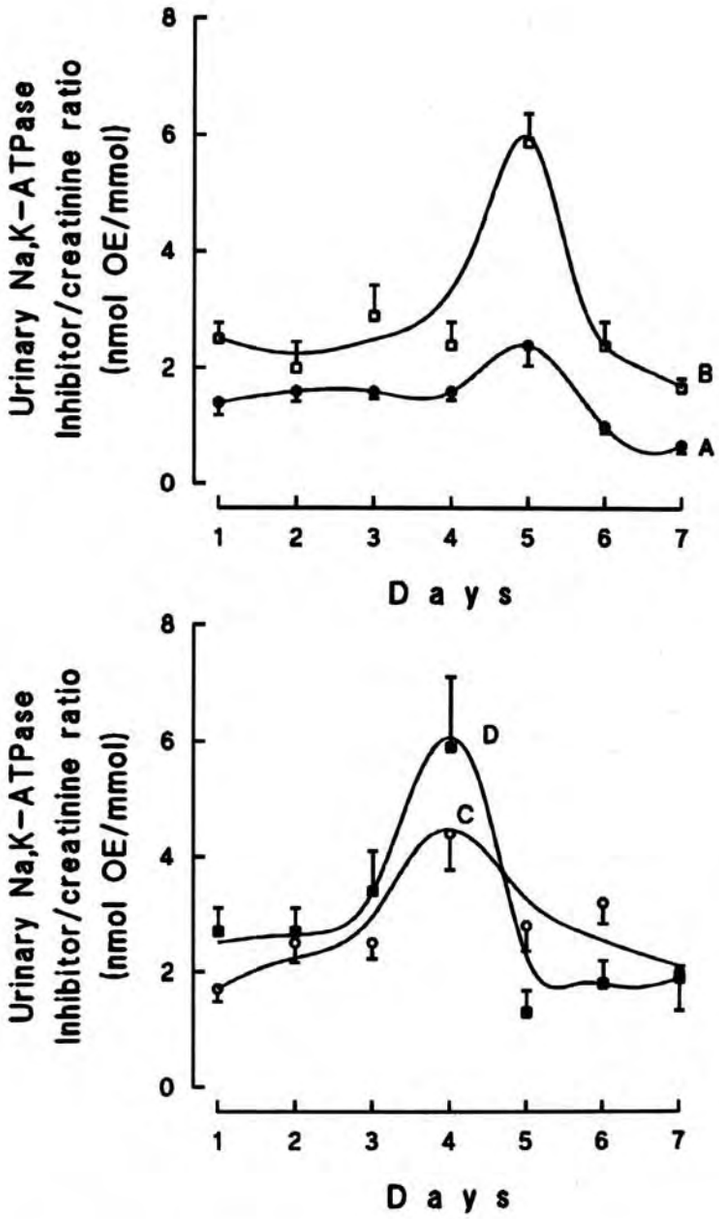


Figure 6-4: Changes of Na, K-ATPase inhibitor/creatinine ratio in the 4 groups of rats.

Group A: low salt
Group B: high salt
Group C: low salt + carbidopa
Group D: high salt + carbidopa
Results as mean ± SEM

DISCUSSION

When renal DA is inhibited by inhibitors of dopa decarboxylase or specific DA receptor anatogonists, sodium excretion has been reported to decrease in man (Ball & Lee 1977, Williams *et al* 1986) and in the rat (Yoshimura *et al* 1987, Shigetomi *et al* 1989). This observation supports the role of renal DA as an important natriuretic factor. However, there are other, conflicting, reports showing that neither dopa decarboxylase inhibitor nor DA receptor anatgonists affect sodium excretion in animal studies (Bass & Murphy 1990). Though DA could be an important natriuretic factor in the kidney when DA is blocked, other natriuretic mechanisms will help to maintain the sodium homeostasis so that effective ECF volume remains unaffected. Reduced natriuresis should only be a temporary phenomenon. Therefore, the inter-relationships between the different natriuretic mechanisms should be studied in detail to understand these conflicting results. It has been postulated that impaired DA mobilization could lead to an increase in ESTI which in turn may initiate the genesis of hypertension (Lee 1987). Elevation of ESTI has been reported in rats treated with metoclopramide (Shigetomi *et al* 1989). The present experiment was designed to study the inter-relationship between DA and ESTI in carbidopa treated rats on high salt diet.

Carbidopa has been used commonly to study the effect of DA on sodium excretion both in man and animals. When the dosage of 40 mg/Kg/day was used in one study of spontaneous hypertensive rats, both sodium and DA excretions were reduced (Yoshimura *et al* 1987). However, there has not been any explanation as to how the dosage of carbidopa was selected. In a preliminary experiment using 40 mg/Kg/day of carbidopa, DA excretion was observed to increase 3 days after the treatment, suggesting that the dosage was not high enough to suppress DA production throughout the study period. Thus, it was decided to use a dose of 120 mg/Kg/day hoping that the 3-fold higher dosage would be enough to inhibit DA production throughout the 7 days. In man, 50 mg carbidopa was reported to inhibit DA excretion for only 1-2 hours (Williams *et al* 1986).

In the present study, carbidopa treatment did not induce significant decrease in sodium excretion (Figure 6-2). Although in the high salt group carbidopa treatment (Group D) gave a slightly lower sodium excretion than in Group B, the difference did not reach statistical significance. Furthermore, sodium excretion in Group D appeared to increase on the last day of the experiment. This result does not agree with the previous observation on carbidopa treatment (Yoshimura *et al* 1987), but is consistent with the report on treatments of rats on normal salt diet on benserazide (Bass & Murphy 1990). The discrepancies in results could be due to biological variation in the animals studied, for example, different animal strains and age of the animals. It could also be due to the type of inhibitor and, for example, dosage, and the duration of treatment. In a study with spontaneous hypertensive rats on high salt diet, the animals were treated with 40 mg/Kg/day of carbidopa for 4 weeks (Yoshimura *et al* 1987). There was no difference in sodium excretion between the experimental group and the control group for the first week of carbidopa treatment. Sodium excretion was lower in the carbidopa treated group only after 1 week. Another study using benserazide for 10 days showed no difference in sodium excretion (Bass & Murphy 1990). In a 7-day study using metoclopramide treatment, sodium excretion was lower in the treated group, but did not reach statistical significance (Shigetomi *et al* 1989). It is possible that several weeks of dopa decarboxylase treatment is necessary before significant decrease in sodium excretion can be demonstrated.

DA excretion in the high salt group (Group B) was significantly higher than in the low salt group (Group A) on day 2 and onwards (Figure 6-3). This result is consistent with the results obtained in section II of this Chapter. A significant correlation between sodium and DA was also observed ($r=0.715$, $p<0.0001$).

DA excretion decreased significantly over the study period in the low salt group (Group A). A similar decrease was also observed in the high salt group after day 5 (Group B). However, the decrease did not reach statistical significance. DA excretion was expressed as DA/creatinine ratio in this study to correct for incomplete collection of urine and any difference in lean body mass between the groups. The rats

used in this experiment were only 1 month old. They were growing and likely to be coupled with an increase in lean body mass, leading to an increase in urinary creatinine excretion. When the rats were kept on a constant salt diet, DA excretion should remain relatively constant. However, the increase in creatinine excretion may explain the apparent decrease in the DA/creatinine ratio. On the other hand, high salt rats had an initial increase in DA excretion for several days before reaching a new steady state. Then, the decrease in DA/creatinine ratio might be delayed by a few days. It is also possible that high salt rats had a reduced rate of increase in body weight due to the increase in salt intake or poor apeptite. This is supported by the smaller increase in body weight. The increase in creatinine excretion will be slower and thus the decrease in DA/creatinine ratio.

There was approximately 70% inhibition of DA on the dosage of carbidopa used in this study. In a study using 40 mg/Kg/day carbidopa treatment in spontaneous hypertensive rats, there was about 60% inhibition of DA excretion after one week of treatment (Yoshimura *et al* 1987a). It appears that a higher dose of carbidopa is required to inhibit DA excretion from day 1 of treatment. However, whether such a dosage leads to other systemic effects has not been reported. The carbidopa treated high salt group (Group D) had the least increase in body weight in this study.

Despite an increase in body weight, and probably an increase in creatinine excretion, there was a trend for DA/creatinine ratio to increase in both carbidopa treated groups. This was more obvious in the high salt group (Group D). Dopa decarboxylase activity is regulated by its substrate L-dopa (Christenson *et al* 1970) and high salt diet has been reported to increase the L-dopa production both in rat (Grossman *et al* 1990) and man (Wolfovitz *et al* 1993). Increase in the substrate will up-regulate the synthesis of dopa decarboxylase. Thus the same dosage of carbidopa will not be sufficient to maintain the inhibition on DA production. The gradual recovery of DA excretion under carbidopa inhibition has been reported both in rat (Yoshimura *et al* 1987, Yoshimura *et al* 1987a) and in man (Ball & Lee 1977, Williams *et al* 1986). Furthermore, when 50 mg of carbidopa was given to healthy males, the inhibition of DA excretion only lasted for 1-2 hours (Williams *et al* 1986). Carbidopa treatment

twice daily may not provide a constant inhibitory effect on renal dopa decarboxylase. Continuous infusion of carbidopa may provide a better model for consistent inhibition of DA production for future studies.

Without carbidopa treatment, the high salt group (Group B) had significantly higher Na, K-ATPase inhibitor excretion than the low salt group (Group A) throughout the study period (Figure 6-4). The pattern of ESTI excretion was similar to that observed on the previous study described in section II of this Chapter. The surge on day 5 was again clearly reproducible. However, a relatively smaller surge was also observed for the low salt rats. The fact that this surge was observed in two different studies makes it unlikely that this is an artifact due to analytical errors.

Carbidopa treatment of both low salt and high salt groups caused significantly higher inhibitor excretion than in the low salt rats without carbidopa (Group A). There was no significant difference between Groups B, C and D. The low salt group on carbidopa treatment (Group C) had similar ESTI excretion to the high salt group. This result suggests that DA may have an inhibitory effect on the production of ESTI. Furthermore, the surge of Na, K-ATPase inhibitor excretion was seen on day 4 for both groups on carbidopa treatment. Again, this result suggests that DA has an inhibitory effect on whatever the signal is that relates to the ESTI surge. The decrease in DA production stimulates this signal to be effective one day earlier. In a study using metoclopramide-treated high salt rats, both plasma and urinary Na, K-ATPase inhibitory activities were significantly higher in the treated rats than the untreated rats (Shigetomi *et al* 1989). However, that study only measured the urinary excretion on days 0, 3 and 7. The surge of inhibitor excretion on days 4 and 5 could, therefore, not be observed. The inhibitor surge had not been reported before and deserves further understanding.

Correlation studies confirmed that DA excretion is significantly correlated with sodium excretion in both groups of high salt rats (Groups B & D). This supports the role of DA being an important natriuretic factor during oral salt loading. With multiple regression analysis, it is shown that almost 50% of sodium excretion can be

attributed to DA. In the presence of carbidopa treatment, there was a significant inverse relationship between DA excretion and ESTI excretion. This may support the hypothesis that ESTI increases when DA production is inhibited (Lee 1987).

The hypothesis tested in this study is that inhibition of DA production during salt loading will cause a much higher ESTI. ESTI increased in control groups given high salt (Group B and Section II). Carbidopa treatment of rats on low salt diet increased ESTI excretion suggesting that under normal circumstances DA is important for natriuresis and that when DA production is inhibited ESTI is secreted to maintain ECF volume (Fig 6-4). This supports the suggestions of Lee. When rats on high salt were treated with carbidopa, ESTI excretion, although higher, was no different from group B (high salt control group). This suggests that either other natriuretic factors come into play or that DA is not important in natriuresis. The observation could also be interpreted to show that ESTI may not be a natriuretic factor or that there is a maximum response above which ESTI cannot increase. ESTI has been suggested to stimulate ANP and vice versa (Crabos *et al* 1988). The observation in this study could be explained as follows: when rats on high salt diet are treated with carbidopa, sodium excretion transiently falls leading to a transient increase in ECF volume which in turn increases ESTI and ANP. These factors bring the ECF volume back to normal.

CHAPTER 7

CONCLUSION

CONCLUSION

Review of the literature shows there are several complex mechanisms to maintain the effective ECF volume. The kidney appears to be the major organ responsible for the regulation of effective ECF volume through the excretion of sodium. There are many sensors, hemodynamic forces and hormonal factors to ensure homeostasis and this presents difficulties in studying the functionial roles of individual factors. It is almost impossible to dissect out an individual factor and study its contribution to sodium excretion. In this thesis, the contributions of two controversial factors, renal DA and plasma ESTI, were studied in greater detail. There have been conflicting reports on the roles of these two factors in natriuresis. Furthermore, the plasma ESTI has been suggested to be a key factor in the pathogenesis of essential hypertension (de Wardener & Clarkson 1985). In this hypothesis, the inability to excrete excess dietary sodium due to an inherited defect in the kidney is the primary feature leading to the development of high blood pressure. Later, it was proposed that this inherited fault is a failure to produce appropriate amounts of renal DA in response to sodium challenge (Lee 1987). The study of these two natriuretic factors provided a unique opportunity not only to understand their contributions to natriuresis, but also to understand the pathogenesis of essential hypertension.

MEASUREMENT OF ESTI

Two methods were developed for the study of ESTI in this thesis. The first one was based on the inhibition of purified Na, K-ATPase and the second one was based on the measurement of DLI. Both methods were applied for the measurement of ESTI in plasma and urine samples.

The method developed for the measurement of Na, K-ATPase inhibitor was based on the automated measurement of Na, K-ATPase activity coupled to PK and LD on a

centrifugal clinical chemistry analyzer. Because of the use of an automated analyzer, the precision of the method was better than other, manual, methods. This method was also efficient as 28 samples can be handled in 15 minutes. By incorporating a 2-hour incubation of the plasma extracts with the enzyme in a potassium-free buffer, the detection limit of the method was lowered to 5 nM, a value similar to that reported for radioreceptor assay (Kelly *et al* 1985). Methods based on the inhibition of purified Na, K-ATPase activity usually require either concentration of the sample, the use of radioactive isotopes or expensive instrumentation. Concentration of biological samples might increase the contribution of other non-specefic inhibitors which are not physiologically important in the regulation of the sodium transport *in vivo* (Woolfson *et al* 1994). With this sensitive method, there was no need for concentration of samples.

The use of solid phase extraction to eliminate protein and electrolyte interference has been commonly used in the measurement of ESTI (page 2-2). However, the analytical performance has rarely been reported. In this thesis, a systematic study was conducted. The introduction of an equlibration time between the sample and the C18 resin improved the precision and recovery. The use of other pretreatment methods, for example boiling and extraction with organic solvents, was studied and found not to be useful. Sep Pak extraction reported in this thesis has been applied for the extraction of ESTI in both plasma and urine samples sucessfully.

Based on coupled enzyme assay Hamlyn et.al (1982) showed a correlation between plasma ESTI concentration and MAP in essential hypertensive patients. Using the method reported in this thesis a correlation between plasma ESTI concentration and systolic blood pressure was observed in a group of hypertensive NIDDM patients (Figure 4-5, page 4-31).

The method developed for the measurement of ESTI by DLI in this thesis was first based on a RIA method. It was later replaced by a HEIA method automated on a centrifugal clinical chemistry analyser. The automation improved the efficiency as well as the precision performance of the method. Improvement in the sensitivity of

the digoxin method has been commonly achieved by altering reaction conditions, such as the antigen and antibody reagent volume ratio (Wijdicks *et al* 1987). In the course of improving the sensitivity of the RIA DLI method, the detection limit was lowered from 0.1 μg/l to 0.03 μg/l (Table 2-8, page 2-46). However, this reaction condition did not improve the detection limit for ESTI in plasma. This finding provides a different perspective on the use of DLI to measure ESTI .

Measurement of DLI in samples was preceded by sample extraction with Sep Pak C 18 resins. This provided a common and convenient preliminary purification method for measuring ESTI by two independent methods. Only 300 μl of plasma sample was required for both assays. Furthermore, as the Sep Pak extraction was optimized for inhibition of Na, K-ATPase activity, the presence of cross-reacting but non-inhibiting species was reduced. Since the Sep Pak eluants were not concentrated, any interference from supra-physiological concentrations of other interfering analytes was redeuced.

There have been conflicting reports on the relationship between Na, K-ATPase inhibitory activity and DLI. In the present study a significant correlation between Na, K-ATPase inhibitory activity and DLI was found in a group of 51 Chinese medical students (Figure 4-1, page 4-5). However, the two methods did not show significant correlations in other studies. It can therefore be concluded that the correlation between Na, K-ATPase inhibitor and DLI depends on both the method of measurement and the origin of the sample.

Since the exact nature of ESTI is not known, it is recommended that a combination of at least two methods be used for its measurement (Goto *et al* 1992). It is desirable for one of the methods to be based on inhibition of cellular sodium transport to simulate the biological actions *in vivo*. The two methods developed in this thesis do not fulfill this criteria. However, only a few studies reported in the literature fulfil this ideal condition. The use of oral rubidium loading to study sodium transport *in vivo* could be considered an ideal method (Boon *et al* 1984).

MEASUREMENT OF URINARY FREE DA

The HPLC method developed for the measurement of urinary free DA was based on the use of ion-pairing reagent in the mobile phase, reverse phase stationary phase and a sensitive ECD. For preliminary purification of the CATS, urine free CATS were adsorbed onto alumina and eluted with 0.1 M HCl. During the process of extraction, the alumina was kept at optimal alkaline pH to ensure good recovery. To protect the CATS from auto-oxidation at alkaline pH, ascorbic acid and sodium metabisulfite were used. Furthermore, to reduce the presence of electrochemical active species in the ECD reaction, ethyl acetate washing was introduced (Davidson & Fitzpatrick 1985). Chromatographic separation of all the three CATS and the internal standard DHBA was achieved in 15 minutes. The developed method is robust and has an analytical performance up to "state of the art".

Due to the intrinic sensitivity of CATS to oxidative agents, preanalytical factors are also important for the reliability of any measurement of urinary free CATS. The use of acid preservatives for urine collection of CATS measurement is common (Table 3-1, page 3-5). In this thesis, a systematic study on the effects of acid concentration, duration and temperature of storage was studied. It was found that HCl was not an ideal preservative for urinary free CATS. 1 M HCl decreased CATS standard solutions by 1-4% in 2 hours at room temperature, probably due to oxidative conversion. Furthermore, the presence of a high acid concentration in the urine samples hydrolyzed the conjugated CATS into free CATS giving falsely high results (Figure 3-6, page 3-42). The rate of hydrolysis is dependent on acid concentration, temperature and duration of storage. 100 ml of 0.5 M HCl in a 2-liter container was found to be a satisfactory preservative based on this systematic study. With this preservative, urinary free DA was stable up to 3 months when stored at −20 °C. However, urinary free NA and A were stable for only up to 1 month (Table 3-9, page 3-48).

Although acid preservatives are still popular, alternative methods for the preservation of CATS in urine samples should be studied in the future. The use of an acidic buffer

solution should provide the optimal pH condition. Addition of antioxidants, like ascorbic acid and sodium metabisulfite, is likely to delay the oxidative conversion process upon storage. Storage of urine samples at lower temperature is also another approach to solve the stability problem.

CROSS SECTIONAL STUDIES IN THE HUMAN

The roles of DA and ESTI in natriuresis were studied in Chinese subjects under physiological conditions by cross-sectional studies. The subjects were allowed to continue their usual diet so that the status of natriuretic factors and sodium homeostasis were all at equilibrium.

In a group of 51 medical students, there was no correlation between sodium excretion and plasma ESTI (page 4-3). In another group of 41 young females, the excretion of DLI did not correlate with sodium excretion (page 4-8). Furthermore, 30% of the variation in DA excretion was accounted for by the variation in sodium excretion. These findings agree with the hypothesis of de Wardener & MacGregor (1983). In healthy normal subjects, the kidney can eliminate excess sodium and there is no stimulus for the production of plasma ESTI. The positive correlation between sodium and DA excretion suggests that there is no abnormality of DA mobilization.

The sodium and DA relationship was also studied in 2 groups of healthy normotensive subjects: a group without a family history of hypertension (Group A) and another group with a family history of hypertension (Group B). Sodium intake was not different between these 2 groups as supported by the insignificant difference in their sodium excretion (Table 4-4, page 4-15). There was a significant correlation between excretion of sodium and DA in Group A, but no correlation in Group B (Figure 4-2, page 4-16). Another group of hypertensive patients (Group C) also did not have a correlation between sodium and DA excretion. The loss of association between

sodium and DA excretion in Group C provides evidence that DA may be an important factor facilitating sodium excretion. Furthermore, the loss of the association in Group B suggests that the abnormality of renal DA production in the hypertensive patient is inherited, not acquired (Lee 1993).

Excretion of DA has been reported to be dependent on age and sex (Gerlo *et al* 1991). In order to understand the relationship between DA and sodium excretion, further study with age- and sex-matched subjects is important. Instead of relying on the family history of hypertension, classification of subjects according to their blood pressure response to salt challenge can be used (Gill *et al* 1988).

DA excretion has been reported to show circadian variation (Kawano *et al* 1990). The DA and sodium relationship could be different between the day and the night. This was shown in oral salt loading studies in this thesis (Figure 5-15, page 5-59). Collection of 24-hour urine samples should be replaced with day and night collections to understand this relationship better.

The importance of renal DA in sodium excretion has been demonstrated by the use of DA receptor antagonists or dopa decarboxylase inhibitor to reduce sodium excretion (Bass & Murphy 1990). However, other effects of these pharmacological manipulation are not well-understood. It has been reported that there is decreased urinary DA in microalbuminuric IDDM and NIDDM patients (Murabayashi *et al* 1989, Patrick *et al* 1990). Thus, 164 NIDDM patients were recruited to study the effects of reduced DA production on sodium excretion under physiological conditions. Both urine collection and venous blood sampling were done twice over a period of 6 weeks in order to obtain a more representative sample from these patients.

The 164 patients were divided into three groups according to UAE. Group A had no albuminuria (UAE <30 mg/d), Group B had microalbuminuria (UAE 30-300 mg/d) and Group C had macroalbuminuria (UAE >300 mg/d). Urine DA excretion decreased inversely with UAE (Table 4-5, page 4-24). However, there was no significant difference between sodium excretion among the 3 groups. This suggests

that sodium balance is maintained by other natriuretic factors. Increased plasma ANP was observed. It may serve as a compensatory mechanism to facilitate sodium excretion. This was supported by their inverse relationships with UAE (Figure 4-3, page 4-25). Increased circulating ANP may increase proteinuria further (Zietse & Schalekamp 1988, Ishi *et al* 1989, Suenaga *et al* 1989, Hirata *et al* 1991).

There was also a dissociation between plasma renin and serum aldosterone concentrations in the NIDDM patients. Serum aldosterone was lower in Group C but plasma renin was higher. The paradoxical increase in plasma renin concentration could be due to a reduced metabolic clearance because of reduced renal function (Rosenberg *et al* 1994). Higher renin concentration may lead to an increased concentration of angiotensin II, which can also reduce DA excretion (Eadington *et al* 1991).

There was a significant increase in MAP with increasing UAE (Table 4-5, page 4-24). MAP of these NIDDM patients correlated significantly with not only age, sodium excretion and ANP, but also with Na, K-ATPase inhibitor (Table 4-6, page 4-28). The patients were also grouped according to their SSBP (Table 4-7, page 4-30). Hypertensive NIDDM patients also had lower DA excretion than the normotensive ones. Again, there was no significant difference in sodium excretion between the two groups. On the other hand, there was significant correlation between the sodium and DA excretion in both hypertensive and normotensive groups. This finding was in contrast to the results observed previously for the hypertensive patients and normotensive subjects with a family of hypertension, in whom the association between sodium and DA excretion did not hold. This suggests that hyertensive NIDDM patients do not have the same genetic defect.

Measurement of the different natriuretic factors in this group of patients provided an insight to the development of hypertension in NIDDM. It can be postulated that, in patients with NIDDM, early renal tubular damage may lead to reduced renal DA production, resulting in impaired sodium excretion. In response to volume expansion and increase in blood pressure, there is a compensatory rise in plasma ANP

concentration. This can facilitate sodium excretion and limit the sodium retention. However, this compensatory phenomenon could also contribute to worsening of albuminuria and possibly renal damage. This may lead to a further reduction in the urinary DA response to salt intake and initiate a vicious cycle involving sodium retention, hypertension and albuminuria. Diminished renal function also leads to the paradoxical rise in renin which may contribute to further reduction in DA production. The increase in plasma Na, K-ATPase inhibitor observed in these patients may contribute to the development of hypertension.

A significant positive correlation between excretion of DA and NA had been observed in all the cross-sectional studies in which urinary CATS were measured. Urinary NA reflected the sympathetic nervous activity. The effect of NA on renal handling of sodium has been reported to be opposite to that of DA. Increased sympathetic nervous activity induces vasoconstriction and increased sodium reabsorption. However, their consistent correlation in these cross-sectional studies suggests that renal DA production is in part under the influence of sympathetic nervous activity. It is possible that renal DA stimulates sympathetic nervous discharge via dopaminergic receptors, or renal NA is a link between sodium intake and tubular DA production. Further studies are required to elucidate the relationship betwen the excretion of sodium, DA and NA.

From this series of cross-sectional studies, there was evidence that DA has a direct association with sodium excretion under physiological conditions. However, in patients with essential hypertension or normotensive subjects with a family history of hypertension, there may an inherited defect in DA mobilization. Reduced production of DA did not result in decreased sodium excretion because of the presence of other natriuretic factors. Plasma ANP, rather than plasma ESTI, appears to be the immediate plasma hormone compensating to facilitate sodium excretion. Longitudinal studies to understand the temporal relationships between DA, ANP, ESTI and renin/aldosterone system in normotensive subjects with a family history of hypertension and in NIDDM patients, should provide further information in the development of hypertension. Furthermore, salt loading experiments on normotensive

subjects with and without a family history of hypertension, and NIDDM patients with different renal DA capacity should give more insights in the roles of DA and ESTI in sodium excretion and their contributions to the development of hypertension.

VOLUME EXPANSION STUDIES IN THE HUMAN

To study the role DA and ESTI in sodium excretion, ECF volume is altered experimentally by headout immersion, saline infusion and oral salt loading. Two oral salt loading studies were performed to simulate events in daily life.

During headout water immersion, it has been shown that there is a highly significant increase in fractional excretion of sodium (Epstein 1992). This suggests that natriuresis by this method of expanding ECF volume is attributable primarily to decreased tubular reabsorption of sodium. A group of 7 Caucassian women undergoing headout water immersion for 3 hours was studied. There were significant increases in plasma ANP and decreases in both renin and aldosterone during the immersion period (Bisson *et al* 1992). No significant changes of both DA and ESTI were observed during the course of immersion (Figure 5-1, page 5-8). On the other hand, there was a 50% reduction in NA excretion indicating that the decrease in sympathetic nervous activity was another driving force for sodium excretion during headout water immersion. Furthermore, the DA/NA ratio increased 250% during the course of immersion (Figure 5-2, page 5-10). Multiple regression analysis showed that sodium excretion correlated inversely with the ratio ($\beta = -0.636$, $p<0.0001$). This result suggested that the decrease in NA, i.e. reduced sympathetic nervous activity, rather than DA, was an important contributor to sodium excretion. Plasma ESTI concentration did not play an important role in this method of ECF volume expansion.

When 4 healthy Chinese males were infused with 1 liter of saline over a period of 2 hours, significant volume expansion was achieved (Table 5-3, page 5-20). Sodium excretion increased more than 2-fold (Figure 5-3, page 5-23). However, there was no significant increase in either DA excretion or plasma ESTI concentration during the course of saline infusion. Although there was a 16% decrease in NA excretion and a 30% increase in the DA/NA ratio, these changes did not reach statistical significance (Figure 5-3, page 5-23). This suggests that the sympathetic nervous system was not involved in the sodium excretion induced by saline infusion. On the other hand, the renin-aldosterone system was suppressed during infusion and may be one of the important factors in facilitating sodium excretion (Figure 5-4, page 5-26). Under the present experimental design, the results suggested that neither DA excretion nor plasma ESTI concentration played significant roles in sodium excretion during saline infusion.

Experimental design of the present saline infusion study could be improved. The number of subjects should be increased. In the present study, subjects were only given 200 ml of water at the start of the infusion. Urine volume decreased significantly during the course of infusion. This was probably due to the increase in plasma osmolality which in turn increases the circulating AVP concentration. AVP is another known factor which can affect sodium excretion. If water were given to maintain sufficient urine flow, the results might have been different. Furthermore, at the end of the infusion period, only 20% of the infused sodium load had been excreted. Urine and blood sampling should be continued for a longer period to monitor the changes of both urine DA excretion and plasma ESTI concentration.

In the first oral salt loading experiment, five healthy Chinese subjects were studied over 9 days. They were on a low salt diet of 40 mmol/d for 3 days, a high salt diet of 390 mmol/d for 3 days, and then on their normal diet of 190 mmol/d for 3 days (Figure 5-6, page 5-40). Sodium excretion was 10-fold higher during the high salt period when compared to the low salt period. Volume expansion was achieved as shown by a significant increase in body weight and decrease in plasma protein concentration (Table 5-7, page 5-39). In this study, the collection of day and night

urine samples was expected to provide more information on the relationship between excretion of DA and sodium as there is a circadian variation of DA excretion. However, there was no signficant increase in either DA excretion or plasma ESTI concentration during this salt loading study in the day or night collections. There was no significant correlation between the sodium and DA excretion. It was recognized that the 3-day period of low salt and high salt might not be sufficient to establish a new steady state for these subjects (Sagnella *et al* 1990). The study period should be extended to at least 5 days as used by other research groups. Furthermore, the subjects should be kept on the same diet to reduce the influence of protein on the production of DA (William *et al* 1986).

The second oral salt loading study was designed to improve on possible experimental design faults that might have contributed to the lack of DA and ESTI response in the first study. Seven young Chinese medical students were recruited. They were kept on the same low-salt hospital diet for 10 days (day -4 to day 5). On day 1, they were given 20 "Slow Sodium" tablets after breakfast. There was a 9-fold increase in sodium excretion between the low and high salt periods (Figure 5-13, page 5-55). Significant ECF volume expansion was achieved as supported by significant suppression of the renin-aldosterone system (Figure 5-14, page 5-58).

A significant increase in plasma ESTI was observed: Na, K-ATPase inhibitor on day 3 and DLI on day 2 (Figure 5-14, page 5-58). This result suggests that the duration of low salt and high salt periods could be critical to induce a significant response of plasma ESTI. Furthermore, there was a dissociation between Na, K-ATPase inhibitor and DLI indicating that the 2 methods were measuring different entities in the plasma samples.

Upon salt loading, sodium excretion reached its new equilibrium on day 3. Plasma ANP increased with salt loading (Figure 5-14, page 5-58). A significant increase of plasma ANP was observed on day 4, and then it decreased abruptly on day 5. Since plasma samples were collected daily before the salt loading, the measured plasma concentration represented the results of the previous day. Plasma ANP peaked when

the sodium excretion reached a new steady state and then it decreased abruptly on the next day. The cause of this decrease in plasma ANP cannot be explained by the results of this study. It is speculated that there could be a maximum limit for the rise of ANP concentration. Infusion studies have shown that ANP induces proteinuria in patients with essential hypertension (Suenaga *et al* 1989) and primary glomerular diseases (Zietse & Schalekamp 1988, Ishi *et al* 1989, Hirata *et al* 1991). Nevertheless, the decrease in ANP could be a contributing factor for the surge of Na, K-ATPase inhibitor on day 5. This phenomenon has not been reported before.

During the low salt period, circadian variation in DA excretion was not observed (Figure 5-15, page 5-59). This suggests that circadian variation of DA excretion was due to sodium intake. Although there was a 23% increase in DA excretion during the day on the high salt diet, this increase did not reach statistical signficance. Furthermore, there was a 10% decrease in DA excretion during the night over the same period. It was interesting to observe that when there was a higher increase in DA excretion during the day, there was a corresponding decrease in DA excretion during the night. Such a phenomenon has not been reported before and deserve further study.

There was no signficant decrease in NA excretion during the 10-day study period (Figure 5-16, page 5-61). However, there was a sharp rise of NA excretion during the night on day 4. This coincided with the decrease in plasma ANP concentration and the increase of plasma Na, K-ATPase inhibitor. It has been reproted that ANP can inhibit the sympathetic nervous system (Goetz 1990). Thus, the inhibitory effects of ANP on the sympathetic system may be a signal for the increase in the production of ESTI. Furthermore, sodium excretion correlated significantly with the DA/NA ratio during day time when the "Slow Sodium" tablets were being taken.

The second oral salt loading experiment provided more insights on the interactions between the different natriuretic factors. One important aspect of the experimental design was to collect blood samples daily and urine samples for both day and night

periods. The frequent sample collection was time consuming and has not been reported in the literature.

From this series of volume expansion studies, it can be concluded that plasma ESTI does not contribute signficantly to sodium excretion during acute volume expansion like headout immersion and saline infusion. On the other hand, it has been demonstrated that plasma ESTI had a significant role in sodium excretion during oral salt loading. With increased dietary sodium intake, expansion of ECF volume can induce increased production of plasma ESTI. When there is a defect in renal excretion of sodium, persistant expansion of ECF volume can lead to increased production of the Na, K-ATPase inhibitor. The result supports de Wardener's hypothesis (de Wardener & Clarkson 1985). On the other hand, DA excretion does not play a significant role in experimentally induced volume expansion. Together with the association between sodium and DA excretion observed in the cross-sectional studies, these results suggest that DA excretion contributes significantly when sodium excretion is at steady state under physiological conditions.

In order to test the hypothesis that a renal fault in DA production leads to the production of ESTI which may be responsible for the pathogenesis of hypertension, sodium loading in a larger number of subjects should be studied. A direct relationship between DA and ESTI will support the hypothesis. Furthermore, inhibition of DA production or inhibition of DA action should show increased production of ESTI. However, the pharmacological effects of the inhibitor or antagonists are not well understood. Thus, NIDDM patients, with a different degree of renal DA capacity, should also show an exaggerated ESTI response. Another possibility is to study normotensive subjects with a family history of hypertension in whom it is likely there is an inherited defect for the DA and sodium relationship. Again, the response of ESTI should be increased.

STUDIES ON THE EFFECTS OF SALT LOADING IN THE RAT

The effects of dietary salt loading in the rat have been studied to examine the temporal relationship between excretion of ESTI and DA. It was found that the excretion of ESTI closely followed the excretion of sodium whereas the excretion of DA lagged behind by a day (Figure 6-1, page 6-5). Furthermore, the observed increase in DA excretion may not be quantitatively adequate to explain the natriuresis. Similar to the observation in the salt loading studies in medical students, a surge in Na, K-ATPase inhibitor excretion was observed on day 5 in the rat. This surge was two days after the sodium excretion reached a new steady state, and also lasted only for 1 day. This phenomenon has not been reported before. Furthermore, this surge was reproducible in the subsequent salt loading study in the rats. Thus, it is unlikely that this is an artifact due to analytical errors. The changes in ANP could be the reason for this increase in ESTI production.

When rats were treated with carbidopa 120 mg/kg/day, there was 70% inhibition of the excretion of DA. However, during salt loading of carbidopa treated rats, there was no significant decrease in sodium excretion compared with the control group (Figure 6-2, page 6-11). This suggests that there are other natriuretic factors maintaining the sodium excretion. Carbidopa treatment of both low salt and high salt groups of rats caused significantly higher Na, K-ATPase inhibitor excretion (Figure 6-4, page 6-13). In the carbidopa treated low salt group, inhibitor excretion was not significantly different from the carbidopa treated high salt group. The result suggests that DA may have an inhibitory effect on the production of ESTI. Furthermore, the surge of Na, K-ATPase inhibitor was seen 1 day earlier on both groups upon carbidopa treatment. This suggests that DA has an inhibitory effect on whatever the signal is that relates to the ESTI surge. The decrease in DA production stimulates this signal to be effective one day earlier. Results of this study support the hypothesis that ESTI increases when DA production is inhibited (Lee 1987).

When rats on a high salt intake were treated with carbidopa, ESTI excretion was not significantly higher than in the corresponding control rats. This suggests either that other natriuretic factors come into play, or DA is not important in natriuresis. The observation could also be interpreted to mean that ESTI may not be a natriuretic factor or that there is a maximum response above which ESTI cannot increase.

In the present carbidopa study, urine NA was not measured due to the presence of other interfering peaks coeluting in some of the urine samples. Similar to the observation made with the salt loading experiments with the medical students, urine NA excretion may provide more insights into the interaction of the DA production and the sympathetic nervous system. Furthermore, changes in circulating ANP concentration may be responsible for the surge of ESTI production. Therefore, ANP concentration in blood should be measured in future studies.

SUMMARY

Both DA excretion and ESTI have been found to be contributing to the process of sodium excretion under different conditions. Under acute volume expansion of ECF volume, ESTI does not play a significant role. DA excretion correlated with sodium excretion under physiological conditions. Changes in different natriuretic factors by experimental manipulation did not affect sodium excretion, probably because of the large number of different mechanisms available to maintain sodium homeostasis. Furthermore, the idea of a maximum concentration for some natriuretic factors like ANP and ESTI, above which further increase is limited, deserves further study.

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analyzed with both the screening tests and by a latex photometric immunoassay (LAPIA; Eiken Chemical, Tokyo, Japan; Poli, Milan, Italy) showing, at a value of 20.3 mg/L, within- and between-batch CVs of 5.6% and 7.8%, respectively. Urines were adjusted to pH 7.0 ±0.1 and stored at −40 °C (*2, 3*).

We found an albumin concentration of 30–200 mg/L for 20 of these subjects—i.e., 16.7%—which compares well with 19.1% and 15.8% obtained respectively by Capps et al. (*1*) and Mogensen (*4*).

With 30 mg/L as our cutoff, both the screening tests had a sensitivity of 100% and the specificity was 95% for Albuscreen, 88% for Microbumintest. Albuscreen had a positive predictive value of 76% and a negative predictive value of 100%; corresponding values for Microbumintest were 62% and 100%. Test efficiency was 95% for Albuscreen, 90% for Microbumintest.

A cutoff set at 20 mg/L modifies the result as follows: sensitivity for Albuscreen was 76%, for Microbumintest 90%; specificities were respectively 99% and 94%. The positive predictive value was 94% and 84% for Albuscreen and Microbumintest whereas the negative predictive value was 93% and 97%, respectively. For both tests, the efficiency was estimated to be 93%. In accord with our results, we tend to prefer 30 mg/L as our cutoff value. In fact, at 30 mg/L we obtain the greatest negative predictive value with both tests, which is particularly relevant when these kits are used as screening tests.

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Assay for Digoxin-like Immunoreactive Substance—Loss of Sensitivity

To the Editor:

Wijdicks et al. (*1*) reported that they have modified the commercial digoxin immunoassay RIANEM TM Digoxin kit (New England Nuclear) to detect lower concentrations of digoxin and, by implication, digoxin-like immunoreactive substance (DLIS) in patients with aneurysmal subarachnoid hemorrhage. They increased the sample volume and reduced the tracer volume to achieve this, and they reported a lower limit of detection, 0.05 μg/L, which they indicated could be lowered still further by manipulation of the reaction condition.

In the course of establishing a more sensitive RIA for measuring DLIS in different biological samples, we attempted to lower the detection limit of the same digoxin kit by increasing the sample volume and varying the tracer and antibody volumes and by prolonging the incubation. The standards 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 μg/L were prepared by diluting the 8.0 μg/L standard with the zero standard included in the test kit. The lower detection limit was assessed by running 10 replicates of the zero standard. A digoxin control was included for every protocol.

Pooled samples of plasma from adults and from cord blood were used to assess the efficiency of detecting DLIS by various protocols. The two samples were pretreated by two different methods to improve detection of DLIS (*2*), namely, the Sep Pak extraction and deproteinization by boiling. The Sep Pak (Waters C_{18} Cartridge) extraction is performed by passing 1 mL of sample through the Sep Pak Cartridge followed by washing with 5 mL of water. DLIS is eluted with 2 mL of methanol, the methanol is evaporated in a vacuum oven, and the residue is reconstituted with 1 mL of the zero standard for the assay. In the second method, the samples are boiled for 10 min, the coagulated protein disrupted, and the mixture centrifuged (20 000 × *g*, 30 min). The supernate is assayed. Table 1 summarizes the protocols used and the results.

The modified protocols did lower the detection limit for digoxin from 0.10 μg/L to 0.03 μg/L. The mean measured digoxin concentration in the quality-control plasma (1.0 μg/L) was 0.96 μg/L (SD 0.032, n = 9, range 0.93–1.00) by all the protocols used. However, the modified protocols detected a lower DLIS concentration compared with the manufacturer's recommended protocol. These results suggest that when the assay is made more sensitive for digoxin, conditions are less favorable for the cross-reacting DLIS. We also observed that the Sep Pak extraction gives higher DLIS results than does the boil–extraction method.

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Table 1. Effect of Modification of Assay Protocol on the Detection Limit for DLIS in Adult and Cord-Blood Plasma

Volume used, μL			Detection limit, μg/L	DLIS detected, μg/L			
				Sep Pak		Boiling	
Sample	Tracer	Antibody		Adult	Cord	Adult	Cord
Incubation time = 30 min							
40[a]	200[a]	200[a]	0.10	0.61	0.86	0.41	0.56
100	200	200	0.03	0.32	0.41	0.33	0.35
100	100	200	0.05	0.24	0.28	0.25	0.27
100	50	200	0.06	0.16	0.23	0.19	0.23
100	200	100	0.09	0.24	0.30	0.18	0.23
100	200	50	0.08	0.29	0.33	0.13	0.31
Incubation time = 1 h							
100	200	200	0.03	0.19	0.30	0.20	0.27
100	100	200	0.03	0.17	0.19	0.18	0.19
100	50	200	0.05	0.04	0.10	0.03	0.10

[a] Manufacturer's recommended protocol with proportionately decreased volume.

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Atrial natriuretic peptide and urinary dopamine output in non-insulin-dependent diabetes mellitus

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1. Disturbances of sodium and water homoeostasis may contribute to the close association between diabetes, hypertension and proteinuria. We therefore studied the patterns of two natriuretic hormones, plasma atrial natriuretic peptide and urinary dopamine, in 165 Chinese patients with non-insulin-dependent diabetes mellitus controlled by diet or oral hypoglycaemic agents on two occasions over a 6-week period. Patients were divided into three groups based on the mean value of two 24 h urinary albumin excretion measurements. In group 1, 88 patients had normoalbuminuria (urinary albumin excretion $\leqq 30$ mg/day), in group 2, 48 patients had microalbuminuria (urinary albumin excretion between 30 and 300 mg/day), and in group 3, 29 patients had macroalbuminuria (urinary albumin excretion $\geqq 300$ mg/day).

2. The supine systolic blood pressure (mean $\pm$ SD) was higher in patients with abnormal albuminuria (group 1: 140.9 $\pm$ 27.4 mmHg; group 2: 158.1 $\pm$ 26.4 mmHg; group 3: 166.7 $\pm$ 23.9 mmHg; $F=13.1$, $P<0.001$, analysis of variance). Urinary sodium output was similar in these three groups of patients. The geometric means (anti-logarithm of 95% confidence interval logarithm) of plasma atrial natriuretic peptide concentrations increased with increasing proteinuria [group 1: 33.3 (29.9–37.1) pg/ml; group 2: 39.1 (34.2–44.6) pg/ml; group 3: 50 (38.6–54.7) pg/ml; $F=4.24$, $P<0.01$; analysis of variance], whereas those of urinary dopamine output were related inversely to proteinuria [group 1: 1291.7 (1167.2–1437.0) nmol/day; group 2: 1142.3 (975.9–1337.2) nmol/day; group 3: 982.7 (775.7–1245) nmol/day; $F=3.10$, $P<0.05$, analysis of variance]. Using stepwise multiple regression analysis, plasma atrial natriuretic peptide concentration ($r^2=0.31$, $F=56.2$, $P<0.001$) was associated with supine systolic blood pressure ($\beta=0.56$, $P<0.001$), which was ($r^2=0.38$, $P<0.001$) related to urinary albumin excretion ($\beta=0.23$, $P<0.001$). In patients with urinary albumin excretion >30 mg/day, plasma atrial natriuretic peptide concentration was negatively correlated with urinary dopamine output ($r=-0.25$, $P<0.02$).

3. Based on these observations, we hypothesize that, in patients with non-insulin-dependent diabetes mellitus, defective dopamine mobilization in the renal tubules may cause impaired natriuresis with increased blood pressure. A compensatory rise in the plasma atrial natriuretic peptide concentration may then contribute to the development of abnormal albuminuria.

INTRODUCTION

There is evidence that patients with insulin-dependent (IDDM) and non-insulin-dependent (NIDDM) diabetes mellitus have an increase in exchangeable body sodium that correlates with their level of blood pressure (BP) [1]. Plasma atrial natriuretic peptide (ANP), a known diuretic hormone [2], has also been reported to be elevated in hypertensive patients [3] and in patients with IDDM and NIDDM [4–8]. Intravenous infusion of ANP, on the other hand, induces proteinuria in patients with essential hypertension [9] and primary glomerular diseases [10–12]. Urinary free dopamine (DA) mobilization in renal tubular cells may be important in promoting natriuresis in response to a salt load [13] via inhibition of Na^+,K^+-ATPase activity [14]. Decreased urinary DA excretion has been reported previously in microalbuminuric patients with IDDM and NIDDM [15, 16]. Based on these previously reported findings, we hypothesize that defective urinary DA excretion secondary to microangiopathic changes in renal tubular cells may contribute to the sodium and water retention, and consequent hypertension in patients with NIDDM. A compensatory rise in plasma ANP

Key words: albuminuria, atrial natriuretic peptide, Chinese, hypertension, non-insulin-dependent diabetes, urinary dopamine output.

Abbreviations: ANP, atrial natriuretic peptide; BP, blood pressure; DA, dopamine; HbA_1, glycated haemoglobin; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; UAE, urinary albumin excretion.

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concentration to promote natriuresis may then contribute to the development of proteinuria in these patients. To examine the possibility of this hypothesis, we have studied the relationships between these two natriuretic hormones, plasma ANP and urinary DA, and BP and albuminuria in a group of Chinese patients with NIDDM.

METHODS

Patients

The study was approved by the Ethical Committee of the Chinese University of Hong Kong. A total of 165 Chinese patients with NIDDM were recruited from the diabetes clinic at the Prince of Wales Hospital, Hong Kong, and were studied on two occasions over a 6-week period. All patients received their usual dietary and oral hypoglycaemic therapy and none was receiving insulin. Antihypertensive treatment was taken by 102 patients, but was withdrawn for at least 2 weeks before the study.

On each of the two study visits, supine (after 5 min of rest) and erect (after 2 min of standing) BP was measured by a single research nurse using a random zero sphygmomanometer. Phase V (disappearance of sound) was taken as the diastolic BP. Venous blood was sampled for measurement of plasma ANP concentration after erect BP was measured. Twenty-four hour urinary excretions of albumin (UAE), DA and sodium were also measured on the two visits. Urinary tract infection was excluded by examination of a midstream specimen of urine. Normoalbuminuria was defined as a mean UAE $\leqq$30 mg/day, microalbuminuria as 30–300 mg/day and macroalbuminuria as $\geqq$300 mg/day being determined on two separate 24 h samples during the 6-week period.

Analyses

Twenty-four hour urine samples for measurement of DA and sodium were collected into acid-containing bottles and were stored at −20°C before analysis. Urine samples for measurement of albumin were collected into plain bottles and were stored at 4°C before analysis within 1 week of collection. Plasma ANP concentration was measured by radioimmunoassay after extraction using Sep-Pak cartridges (Waters Chromatography Division, Millipore Corporation, Milford, PA, U.S.A.) as described previously [17]. Intra- and inter-assay coefficients of variation were both between 11 and 14% at varying concentrations of ANP in human plasma. The detection limit was 10 pg/ml. Urinary free DA was extracted on to alumina [18] and quantified by h.p.l.c. with electrochemical detection based on previously published methodology [19] with a modification of the composition of the mobile phase, i.e. 50 mmol/l sodium acetate from BDH (Poole, Dorset, U.K.), 20 mmol/l citric acid from Sigma (Poole, Dorset, U.K.), 4 mmol/l 1-octanesulphonic acid from Sigma, 2 mmol/l triethylamine from Merck (Darmstadt, Germany) and 0.1 mmol/l EDTA from BDH were dissolved in 1 litre of Milli-Q water/methanol (86:14, v/v). The mobile phase was filtered by a 0.45 μm filter and was degassed before application. The flow rate was 2 ml/min. The intra-assay and inter-assay coefficients of variation were 1.27% and 8.7% respectively. The mean recovery of urinary free DA was 91.1%, and the detection limit was 3 nmol/l. Urinary albumin concentration was determined by immunoturbidimetry [20] with intra-assay and inter-assay coefficients of variation of 3.3% and 6.7%, respectively. The detection limit was 2.5 mg/l. Urinary sodium concentration was measured by indirect ion-selective electrodes on the parallel multi-channel analyser (American Monitor). Plasma and urinary creatinine concentrations were measured by the Jaffe method on a Beckman Astra-8 Clinical Chemistry Analyser (Beckman Instruments, Fullerton, CA, U.S.A.). Glycated haemoglobin (HbA_1) was measured by gel electrophoresis (Ciba Corning Diagnostics Corp., Alto, CA, U.S.A.) with a normal reference range between 6.5 and 8.5%. Plasma fructosamine concentration was measured by published methodology [21], and fasting plasma glucose concentration by a glucose oxidase method (Reagent Kit; Diagnostic Chemicals Ltd).

Statistical analysis

The mean values of all variables obtained on both visits were used for analysis. The distributions of urinary albumin, sodium and DA outputs and plasma ANP concentration were positively skewed and were log transformed. Their results were expressed as geometric means (anti-logarithms of 95% confidence interval logarithms); other results were expressed as means ± SD. Correlation coefficients were calculated by least squares analysis. Stepwise multiple regression analysis was used to study the interrelationships between these variables. Comparisons between the patient groups were performed using analysis of variance and Student's *t*-test, as appropriate. A P value of less than 0.05 (two-tailed) was considered to be statistically significant. Bonferonni's Inequality Theorem was used to adjust the P value for multiple group comparisons.

RESULTS

The clinical features of the patients are shown in Table 1. Based on the mean value of two measurements of UAE, 88 patients had normoalbuminuria (UAE $\leqq$30 mg/day), 48 patients had microalbuminuria (UAE between 30 and 300 mg/day) and 29 patients had macroalbuminuria (UAE $\geqq$300 mg/day). The supine systolic BP was higher in patients

Table 1. Clinical features and laboratory data of the patients with NIDDM. Values are means ± SD. Statistical significance (analysis of variance): *$P<0.05$, †$P<0.001$.

	Normoalbuminuria (UAE ≦ 30 mg/day)	Microalbuminuria (UAE 30–300 mg/day)	Macroalbuminuria (UAE ≧ 300 mg/day)
Age (years)	52.6 ± 10.0	57.0 ± 11.9	54.4 ± 12.2
Body wt. (kg)	60.7 ± 10.4	62.8 ± 12.9	60.4 ± 11.3
Supine BP (mmHg)	141 ± 27/83 ± 12	159 ± 27/87 ± 14	167 ± 24†/87 ± 10
Erect BP (mmHg)	142 ± 29/88 ± 13	159 ± 27/92 ± 13	160 ± 24†/85 ± 13*
Plasma creatinine concn. (μmol/l)	76.3 ± 16.3	80.3 ± 19.6	98.3 ± 35.8†
HbA_1(%)	10.2 ± 1.7	10.7 ± 2.1	10.7 ± 1.6
Plasma fructosamine concn. (mmol/l)	2.34 ± 0.30	2.39 ± 0.27	2.22 ± 0.25*
Fasting plasma glucose concn. (mmol/l)	8.4 ± 2.4	8.9 ± 2.3	9.1 ± 2.6

with abnormal albuminuria (normoalbuminuria: 140.9 ± 27.4 mmHg; microalbuminuria: 158.1 ± 26.4 mmHg, macroalbuminuria: 166.7 ± 23.9 mmHg; $F=13.1$, $P<0.001$, analysis of variance). Urinary sodium output was similar in all three groups of patients. Whereas the plasma ANP concentration increased with increasing proteinuria [normoalbuminuria: 33.3(29.9–37.1) pg/ml; microalbuminuria: 39.1(34.2–44.6) pg/ml; macroalbuminuria: 50 (38.6–54.7) pg/ml; $F=4.24$, $P<0.01$, analysis of variance], urinary DA output was related inversely to proteinuria [normoalbuminuria: 1291.7(1167.2–1437.0) nmol/day; microalbuminuria: 1142.3(975.9–1337.2) nmol/day; macroalbuminuria: 982.7(775.5–1245) nmol/day; $F=3.10$, $P<0.05$, analysis of variance]. The P values for between-group comparisons are shown in Fig. 1.

Supine systolic BP correlated significantly and positively with plasma ANP concentration ($r=0.57$, $P<0.001$) and negatively with urinary DA output ($r=-0.25$, $P<0.01$, Fig. 2). UAE was related in a positive fashion to plasma ANP concentration ($r=0.31$, $P<0.001$) and negatively to urinary DA output ($r=-0.18$, $P=0.025$, Fig. 2). Urinary DA output was significantly related to urinary sodium output whether the patients were analysed as a whole ($r=0.51$, $P<0.001$) or divided into those with UAE ≦ 30 mg/day ($r=0.50$, $P<0.001$) or with UAE > 30 mg/day ($r=0.54$, $P<0.001$, Fig. 3).

Table 2 shows the correlation matrix between age, body weight, glycaemic control, renal function, UAE, plasma ANP concentration, BP, urinary sodium output and urinary DA output. Since some of these variables were inter-correlated, we further used stepwise multiple regression analysis to predict the most significant relationship and the results are summarized in Table 3.

There was a weak inverse relationship between plasma ANP concentration and urinary DA output ($r=-0.15$, $P=0.056$). The correlation coefficients between plasma ANP concentration and urinary DA output were negative in both microalbuminuric ($r=-0.25$, not significant) and macroalbuminuric ($r=-0.25$, not significant) patients, although the relationship became significant only when both

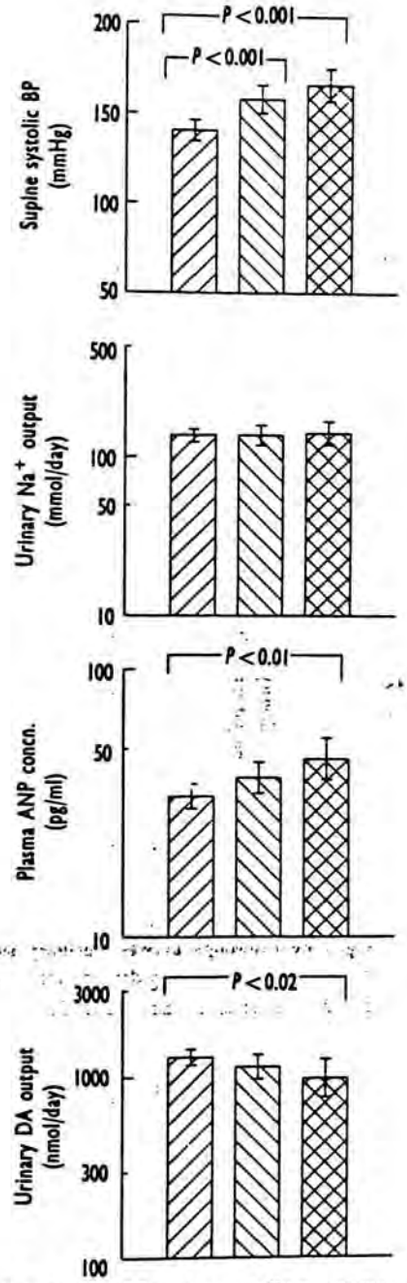

Fig. 1. Supine systolic BP, urinary sodium output, plasma ANP concentration and urinary DA output in patients with normo- (▨) micro- (▧) and macro- (▩) albuminuria. Values are means with 95% confidence intervals shown as error bars. All values, except supine systolic BP, are expressed on logarithmic scales. The adjusted P values for between-group comparisons, considered to be significant at 0.016, are also shown. See the text for P values using analysis of variance.

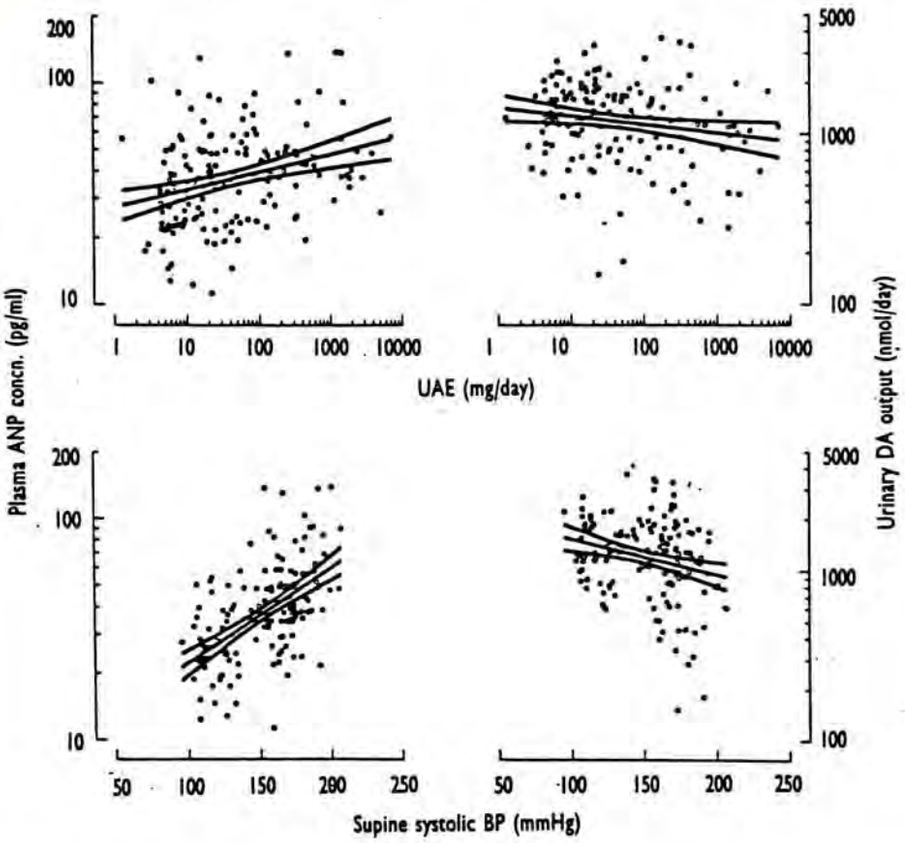

Fig. 2. Relationships between supine systolic BP, UAE, urinary DA output and plasma ANP concentration in 165 patients with NIDDM. All values, except supine systolic BP, are expressed on logarithmic scales. The regression line and 95% confidence intervals are also shown. See the text for correlation coefficients.

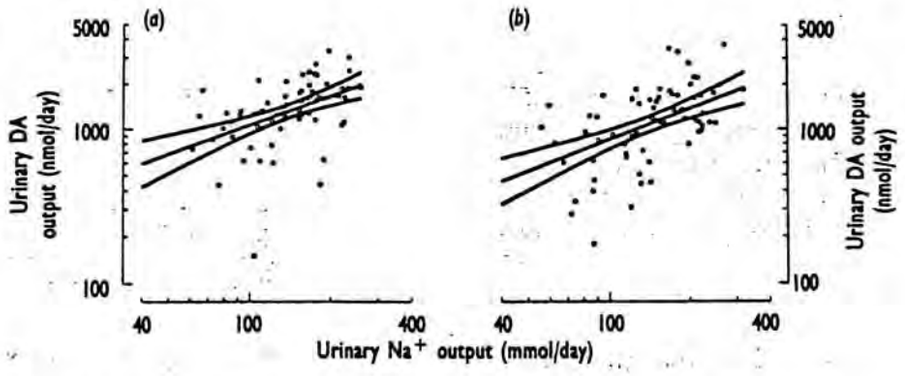

Fig. 3. Relationships between urinary sodium and DA outputs in patients with normal (UAE ≦ 30 mg/day, *a*) and abnormal (UAE > 30 mg/day, *b*) albuminuria. All values are expressed on logarithmic scales. The regression line and 95% confidence intervals are also shown. See the text for correlation coefficients.

groups (UAE > 30 mg/day) were analysed as a whole ($r = -0.25$, $P = 0.027$, Fig. 4).

DISCUSSION

The nature of the relationships between hypertension, diabetes mellitus and albuminuria remains ill-understood, although a recent report indicates that persistent microalbuminuria precedes the increase in arterial BP in patients with IDDM [22]. There is evidence suggesting that an increased exchangeable body sodium might contribute to the development of hypertension among patients with IDDM and NIDDM. Possible mechanisms for the volume expansion and hypertension in these patients include, among others, enhanced proximal tubular sodium–glucose co-transport owing to hyperglycaemia and the anti-natriuretic effects of high circulating levels of insulin [1, 23].

Plasma ANP is primarily secreted from the atrial tissues in response to atrial distension due to plasma volume expansion [2]. Increased plasma ANP levels have been previously reported in patients with essential hypertension [3], and recently in patients with IDDM and NIDDM [4–8]. The actions of plasma ANP include diuresis,

Table 2. Correlation matrix showing the correlation coefficients between age, body weight, BP, glycaemic control, renal function, plasma ANP concentration and urinary sodium and DA outputs in patients with NIDDM. Abbreviation: NS, not significant. Statistical significance: *$P<0.05$, †$P<0.01$, ‡$P<0.001$, §$P=0.056$.

	Age	Body wt.	Supine systolic BP	Plasma glucose concn.	Plasma fructosamine concn.	HbA_1	Plasma creatinine concn.	Plasma ANP concn.	Urinary DA output	Urinary NA^+ output	UAE
Age	1										
Body wt.	−0.3‡	1									
Supine systolic BP	0.51‡	−0.18*	1								
Plasma glucose concn.	−0.15*	NS	NS	1							
Plasma fructosamine concn.	NS	NS	NS	0.58‡	1						
HbA_1	NS	NS	NS	0.70‡	0.68‡	1					
Plasma creatinine concn.	0.3‡	NS	0.23†	−0.17*	NS	−0.2†	1				
Plasma ANP concn.	0.32‡	NS	0.57‡	NS	NS	NS	0.16*	1			
Urinary DA output	−0.29‡	0.33‡	−0.25†	NS	NS	NS	−0.37‡	−0.15§	1		
Urinary Na^+ output	−0.24†	0.35‡	NS	NS	NS	NS	NS	NS	0.51‡	1	
UAE	NS	NS	0.42‡	NS	NS	NS	0.28‡	0.31‡	−0.18*	NS	1

Table 3. Inter-relationships between age, body weight, BP, glycaemic control, renal function, plasma ANP concentration and urinary sodium and DA outputs in patients with NIDDM using stepwise multiple regression analysis. Abbreviations: β, standarized regression coefficient; NS, not significant. Statistical significance: *$P<0.05$, †$P<0.01$, ‡$P<0.001$.

Dependent variables...	Plasma ANP concn.	Urinary DA output	UAE	Supine systolic BP
Analysis of variance for regression model...	$r^2=0.31$ $F=56.2$‡	$r^2=0.38$ $F=20.3$‡	$r^2=0.30$ $F=14.0$‡	$r^2=0.48$ $F=39.2$‡
Independent variables				
Age	NS	NS	NS	$\beta=0.38$‡
Body wt.	NS	$\beta=0.16$*	NS	NS
Supine systolic BP	$\beta=0.56$‡	$\beta=0.15$*	$\beta=0.33$‡	—
Plasma glucose concn.	NS	NS	$\beta=0.45$‡	NS
Plasma fructosamine concn.	NS	NS	$\beta=-0.38$‡	NS
Plasma creatinine concn.	NS	$\beta=-0.30$‡	$\beta=0.23$†	NS
Plasma ANP concn.	—	NS	NS	$\beta=0.39$‡
Urinary DA output	NS	—	NS	NS
Urinary Na^+output	NS	$\beta=0.39$‡	NS	NS
UAE	NS	NS	—	$\beta=0.23$‡

natriuresis and vasodilatation. In conjunction with the renin–angiotensin–aldosterone system, ANP modulates the vascular tone and renal excretory functions in man [2]. In addition, intravenous infusion of ANP has been reported to induce proteinuria in patients with essential hypertension [9] and primary glomerular diseases [10–12]. A rise in intraglomerular vascular resistance owing to postglomerular vasoconstriction and increased renal blood flow may explain the increase in glomerular filtration rate and filtration fraction after ANP infusion [24, 25].

A disproportionately high urinary free DA concentration which cannot be explained by the renal clearance of plasma DA was the first observation that led to the renal sodium–DA hypothesis. Experimental and clinical studies suggest that L-dopa is actively taken up and converted to DA by L-dopa decarboxylase in the renal tubular cells. This uptake process is dependent on the sodium concentration in the proximal tubule [26]. The process of natriuresis has been shown to be facilitated by the inhibition of Na^+, K^+-ATPase activity by DA synthesized intra-renally [14, 27, 28].

It has been suggested that early microangiopathic changes within the renal tubules may initiate and exacerbate diabetic nephropathy [29]. The link between these early structural changes and the development of proteinuria and hypertension is likely to be multifactorial and complex, but may involve an interplay between defective renal mobilization of DA in response to sodium loading which leads to expansion of the extracellular fluid volume, increased arterial BP and ANP levels, which,

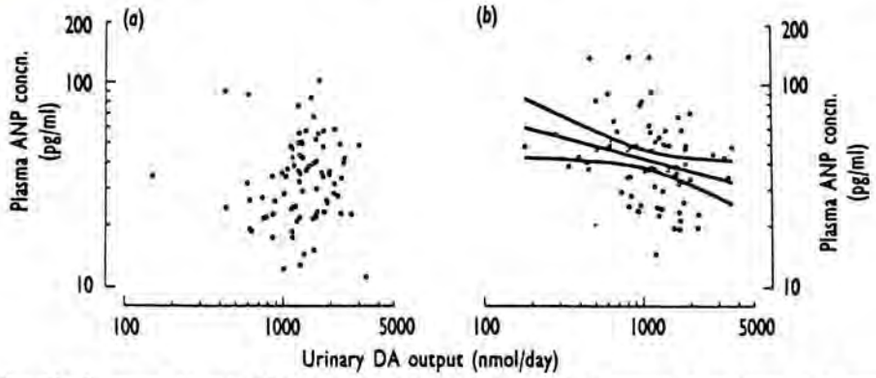

Fig. 4. Relationship between plasma ANP concentration and urinary DA output in patients with normal (UAE ≦ 30 mg/day, *a*) and abnormal (UAE > 30 mg/day, *b*) albuminuria. All values are expressed on logarithmic scales. The regression line and 95% confidence intervals are also shown. See the text for correlation coefficients.

together and individually, may contribute to proteinuria and progressive structural damage within the kidneys.

In this regard, we observed a highly significant relationship between urinary sodium and DA outputs (Fig. 3), which is in accord with the renal sodium–DA hypothesis. In addition, there was an overall trend for the urinary DA excretion to decline with increasing proteinuria (Fig. 2). Defective DA mobilization in response to salt loading has been reported in patients with chronic renal failure [30], whereas others have reported low levels of urinary DA excretion in microalbuminuric patients with IDDM [15] and NIDDM [16]. Furthermore, despite controlling for other confounding factors, we observed that the plasma ANP concentration is strongly correlated with BP which relates to UAE. Taken in conjunction with these correlations, the negative relationship between urinary DA excretion and plasma ANP concentration in patients with abnormal albuminuria (Fig. 4) suggests the possibility that defective DA mobilization in patients with abnormal albuminuria might lead to a compensatory rise in plasma ANP concentration in order to promote natriuresis.

Based on these clinical observations, we hypothesize that, in patients with NIDDM, early renal tubular damage may result in impaired sodium excretion, in part through defective DA mobilization, and hence contribute to the development of hypertension. A compensatory rise in plasma ANP concentration in response to plasma volume expansion and to hypertension may limit the renal sodium retention, but could also contribute to the development of albuminuria and possibly renal damage. This may lead to a further reduction in the urinary DA response to salt intake and initiate a vicious cycle involving sodium retention, hypertension and albuminuria. The individual elements contributing to this hypothesis have support from other clinical and experimental studies. However, further dynamic tests examining the responses of plasma ANP and urinary DA to salt loading and their relationship with BP and proteinuria in diabetic patients are required in order to clarify and extend our clinical observations.

ACKNOWLEDGMENTS

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EFFECTS OF ORAL SODIUM LOADING ON THE URINARY EXCRETION OF CATECHOLAMINES IN NORMOTENSIVE CHINESE

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The renal excretion of noradrenaline (NA) and dopamine (DA) and the interrelationships between urinary sodium and DA in response to oral sodium loading (unrestricted sodium intake + 200 mmol/day) for 3 days were studied in 5 healthy Chinese subjects without a family history of hypertension. There was a five- to eightfold increase in urinary sodium outputs during the high sodium intake period ($p < 0.001$). There was no significant increase in urinary DA outputs in response to the salt load and thus no correlation between urinary sodium and DA excretion. This is in direct contrast to previous findings in Caucasian normotensive subjects. A bigger study is needed to confirm or refute if a reduction in sympathetic activity is the predominant mechanism for dealing with increased salt intake in Chinese.

KEY WORDS: Dopamine, noradrenaline, sodium loading, urine.

INTRODUCTION

Natriuresis following sodium loading is partly mediated by increased production of dopamine (DA) in the kidney which is reflected by an increase in urinary DA excretion. This has been demonstrated in man (Oates *et al.*, 1979) and animals (Ball *et al.*, 1978). Lee (1981) suggested that an impaired DA response to salt loading may lead to hypervolaemia and hypertension in some individuals. Urine DA failed to increase with salt loading in hypertensive patients (Harvey *et al.*, 1984) and this appeared to occur in salt-sensitive rather than salt-resistant hypertensives (Gill *et al.*, 1988). Subsequent cross-sectional studies in different ethnic groups have demonstrated that Caucasians and Thais show a strong positive correlation between 24-hour urinary sodium and DA outputs whereas other groups such as Iranians and black West Africans do not (Critchley *et al.*, 1989). This could indicate that the ethnic groups lacking the sodium-DA relationship may not show a renal DA response to salt loading and thus be more prone to salt-sensitive hypertension or else have another predominant mechanism for dealing with increased salt intake. Cross-sectional studies in normotensive Chinese subjects have also shown a positive correlation between urinary sodium and DA (Chan *et al.*, 1991) so it was anticipated that normal Chinese subjects would respond to oral sodium with increase in urinary DA similar to Caucasians.

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Another mechanism which appears to contribute to the natriuresis following sodium loading is a reduction in sympathetic activity which can be demonstrated by reduction in urinary noradrenaline (NA) excretion (Alexander *et al.*, 1974; Weinberger *et al.*, 1982; Castellano *et al.*, 1986; Gill *et al.*, 1988). The present study was a preliminary investigation into the role of these two mechanisms in dealing with oral salt loading in healthy Chinese subjects.

MATERIALS AND METHODS

Five normotensive, healthy, ambulatory Chinese subjects without a family history of hypertension (4 men and 1 woman, aged 23 to 35 years) were studied. All the participants gave informed consent.

During the 9-day study period, alcohol and medications were avoided. All 5 subjects were on a low salt diet (70 mmol sodium) provided by the hospital kitchen from days 1 to 3, and on their normal diet with unrestricted sodium intake from days 4 to 9. In addition, they took 20 'Slow Sodium' tablets (10 tablets at 9 a.m. and 1 p.m.) on days 4–6 (high salt intake).

Subjects were weighed and their blood pressure measured after sitting for 5 minutes using a Hawksley random-zero sphygmomanometer at 8 a.m. before breakfast on days 4 and 7. Mean arterial pressure (MAP) was calculated by adding one-third of the pulse pressure to the diastolic pressure (phase V).

From days 1 to 9, all subjects collected their urine for 24 hours into plastic bottles containing 25 ml 5 M HCl. Aliquots were refrigerated at −20°C until assayed for DA, NA, creatinine, sodium and potassium.

Urinary free DA and NA were determined by HPLC with electrochemical detection (Davidson and Fitzpatrick, 1985) after extraction onto alumina. The intra-assay and inter-assay variabilities of this assay were 3 and 9% for DA and 1.5 and 10% for NA, respectively. Urinary sodium and potassium were measured by ion-selective electrodes and creatinine by the Jaffe reaction on a Beckman Astra-8 Analyzer (Beckman Instruments, USA).

All the data were expressed as means ± SEM. The urine collection on the last day of the low sodium diet (day 3) was chosen as the baseline. Changes in urinary volume, sodium, potassium, DA, and NA were analyzed with respect to time (from days 3 to 6) using multivariate analysis for repeated measures (MANOVA). Significance of differences for all variables when compared with the baseline were assessed by Wilcoxon signed-rank test. Correlation coefficients between urinary excretion of DA and that of sodium were determined by simple regression analysis. *P* values of less than 0.05 were considered statistically significant.

RESULTS

The 5 subjects showed changes in their MAP from −17.2 to 1.6% but the overall average was not significantly different from baseline (Table 1); they were therefore regarded as salt-resistant (Gill *et al.*, 1988). All the subjects showed increases of 0.3 to 2.8% (mean 1.0%, $p<0.05$) in body weight after high sodium intake for 3 days.

The mean daily excretion of sodium, potassium, creatinine, DA and NA and the urine volume during the 9-day study period are shown in Table 2 and Figure 1.

TABLE 1
Effect of sodium intake on blood pressure (systolic, diastolic and mean arterial) and body weight in 5 healthy Chinese subjects

	Low sodium intake (70 mmol/day	High salt intake (free diet + 200 mmol/day)
Blood pressure (mmHg)		
Systolic	98±2	100±3
Diastolic	67±4	59±3[a]
Mean arterial	78±2	73±3
Body weight (kg)	56.1±2.7	56.7±2.7[a]

[a] $p<0.05$ by Wilcoxon signed-rank test when compared with low sodium intake.

TABLE 2
The 24-hour urine volume and urinary excretion of sodium, potassium, creatinine, dopamine and noradrenaline following oral salt loading (days 4–6)

Days	Volume (l)	Sodium (mmol)	Potassium (mmol)	Creatinine (mmol)	Dopamine (nmol)	Noradrenaline (nmol)
1	2.1±0.3	109±12	46.6±3.4	12.1±1.4	1077±57	128±11
2	1.6±0.3	66±8	42.8±4.0	13.4±1.9	1318±207	170±27
3	1.2±0.2	40±3	38.9±2.8	12.2±1.3	1284±243	158±34
4	1.5±0.2	216±37[b]	67.8±3.4[b]	13.6±1.7[b]	1408±57	179±11
5	2.3±0.3[b]	382±43[b]	60.7±6.6[b]	13.3±2.1	1379±180	164±32
6	2.2±0.5[b]	389±39[b]	40.0±3.7	11.8±1.5	1298±265	121±33
7	2.8±0.5[b]	317±53[b]	39.5±2.8	12.4±1.2	1481±265	174±14
8	1.8±0.3	237±45[b]	49.9±9.8	14.9±3.1	1670±168	197±20
9	1.9±0.2[b]	183±36[b]	40.0±1.4	12.6±1.5	1528±182	205±26

[a] Values are means±SEM.
[b] $p<0.05$ by Wilcoxon signed-rank test when compared with day 3 on low salt.

There was a five- to eightfold increase in urinary sodium output during the high sodium intake period ($p<0.001$; MANOVA) and peak sodium excretion was seen on day 6. Mean sodium excretion gradually fell to around 180 mmol/day by days 8–9 after stopping sodium supplementation. Potassium excretion and urine volume also increased ($p<0.05$; MANOVA) with the sodium loading on days 5–7 and 4–5, respectively (see Table 2).

There were no significant changes in urinary DA ($p<0.5$; MANOVA) or NA ($p<0.5$; MANOVA) outputs during high sodium intake. At the time of peak sodium excretion (on day 6), the changes in NA excretion varied from −77.2 to 16.5% (mean −30%) when compared with baseline and the changes in DA excretion were from −20.4 to 23.6% (mean 0.6%). However, both changes did not reach statistical significance. As a whole, urinary excretion of DA showed a similar pattern to that of NA throughout the study (Figure 1).

There was no significant relationship between urinary sodium and DA ($n=45$, $r=0.21$, $p>0.1$). However, a positive correlation between urinary NA and DA outputs was seen ($n=45$, $r=0.83$, $p<0.001$).

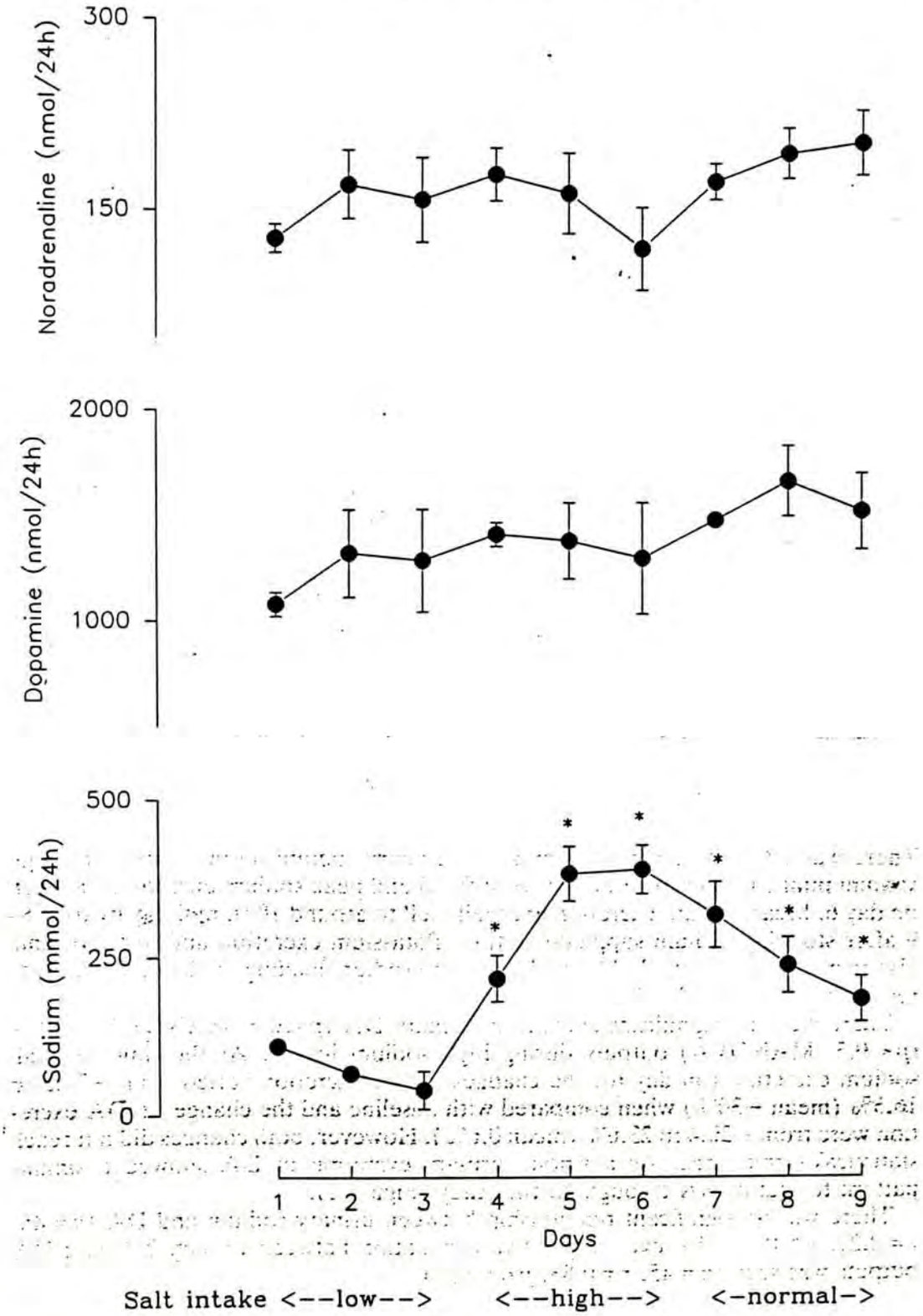

FIGURE 1 Group means ± SEM of urinary sodium, dopamine and noradrenaline outputs in response to oral sodium loading in 5 healthy Chinese subjects. $*p<0.05$ when compared with baseline (day 3).

DISCUSSION

Dietary sodium loading and the acute infusion of isotonic saline have been widely used to determine the factors controlling natriuresis in health and disease. An increase in renal DA output (Alexander *et al.*, 1974; Weinberger *et al.*, 1982; Castellano *et al.*, 1986) and a reduction in the activity of the sympathetic nervous system (Alexander *et al.*, 1974; Weinberger *et al.*, 1982; Castellano *et al.*, 1986; Gill *et al.*, 1988) and of the renin–angiotensin–aldosterone axis (Coghlan *et al.*, 1980) would all be expected to facilitate sodium excretion. In addition, there are suggestions that atrial natriuretic peptide (Laragh, 1985; Sagnella *et al.*, 1985) and renal prostaglandins (Lifschitz *et al.*, 1978; Epstein *et al.*, 1979) play important roles in the physiological regulation of sodium excretion. In the present study, we investigated the role of two of these factors in modulating natriuresis in healthy Chinese subjects during periods of different sodium intake.

Our data demonstrate that a high sodium intake (unrestricted sodium diet + 200 mmol/day) for 3 days was not associated with an increase in MAP in these normal Chinese subjects. Unlike the studies on healthy Caucasian subjects in whom 50–100% increases in urine DA was seen (Alexander *et al.*, 1974; Lee, 1986; Gill *et al.*, 1988; Goldstein *et al.*, 1989), we did not find a significant increase in urine DA following salt loading. Although the present study might be too small to show a DA response of smaller magnitude, the lack of a DA response in this small group of Chinese subjects is also supported by the absence of a significant correlation between urinary sodium and DA outputs. This is in contrast to our findings from a cross-sectional population study of healthy Chinese subjects without a family history of hypertension in whom a significant urinary sodium/DA correlation was seen (Chan *et al.*, 1991).

It is also worth noting that at the time of peak sodium excretion (day 6), a 30% reduction in urinary NA output was seen, although such a change did not reach statistical significance. As a reduction in sympathetic activity is known to facilitate sodium excretion (Alexander *et al.*, 1974; Weinberger *et al.*, 1982; Castellano *et al.*, 1986; Gill *et al.*, 1988), this might well be the predominant mechanism for dealing with increased salt intake in the Chinese. However, a bigger study is required to confirm this hypothesis.

We have previously reported highly significant positive correlations between urinary NA and DA outputs in a cross-sectional study of normotensive and hypertensive subjects (Chan *et al.*, 1991) and this was confirmed in the present study. The significance of this positive relationship between urinary free NA and DA is not clear.

In conclusion, high sodium intake for 3 days was not associated with an increase in MAP or urinary DA output in normotensive Chinese subjects. A bigger study is needed to confirm if a reduction in sympathetic activity is associated with increased salt intake in Chinese subjects.

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CARDIO 01667

Dietary electrolytes and urinary natriuretic factors

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We examined the relationship between the excretion of electrolytes (sodium, potassium and calcium), dopamine and digoxin-like immunoreactive substance in 41 young healthy female subjects (age 18–23 years) in order to study the interaction of electrolyte intake on dopamine and digoxin-like immunoreactive substance—factors which have been postulated to have a pathogenic role in hypertension. Sodium excretion was significantly correlated with dopamine excretion ($r = 0.545$, $P < 0.0005$) and digoxin-like immunoreactive substance ($r = 0.359$, $P < 0.02$). There was also a significant correlation between calcium and digoxin-like immunoreactive substance ($r = 0.345$, $P < 0.03$). Stepwise multiple regression analysis further confirmed that sodium is the only contributor to dopamine excretion and calcium is the only contributor to digoxin-like immunoreactive substance ($r^2 = 0.114$). We conclude that in young healthy subjects dopamine excretion is determined partly by sodium intake and that the excretion of digoxin-like immunoreactive substance is independent of sodium intake.

Key words: Natriuresis; Calcium intake; Sodium intake; Digoxin-like immunoreactive substance

Introduction

Dietary intake of sodium, potassium and calcium has been suggested to be involved in the pathogenesis of hypertension [1]. Epidemiological studies show that blood pressure is directly related to sodium intake and inversely related to potassium intake [2–7]. Potassium depletion increases blood pressure in hypertensive [8] as well as normotensive subjects [9]. A low potassium intake has been suggested to be an important risk factor for the development of hypertension [10]. Hypertensive subjects have been reported to ingest less calcium than normal subjects and abnormalities in calcium homeostasis have been described in hypertensives [1]. The mechanism of action of these nutrients on raising blood pressure is not clear. It has been proposed that in 'susceptible' individuals the ability of the kidney to excrete salt is impaired. As a result sodium retention occurs and the ensuing volume expansion stimulates the release of a natriuretic substance which causes inhibition of sodium pump [11]. In vascular smooth muscle, inhibition of sodium pump leads to hypertension via a change in intracellular calcium [12,13]. Dietary potassium and calcium may act through the same mechanism [8]. Calcium supplementation has been shown to cause a reduction in plasma concentration of sodium transport inhibitor [14]. It has been shown that dopamine is a natriuretic factor and a failure to mobilise dopamine may be the renal 'abnormality' seen in

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salt sensitive hypertensives [15–18]. The Na^+, K^+ ATPase inhibitor (sometimes called digoxin-like immunoreactive substance) has been measured using its cross reactivity to digoxin antibodies. We postulated that if dopamine and digoxin-like immunoreactive substance were directly or indirectly regulated by dietary intake of sodium, potassium and calcium then there should be a relationship between the excretion of these electrolytes and the excretion of dopamine and digoxin-like immunoreactive substance. In this study we have examined the relationship between these variables in a group of young healthy female subjects.

Materials and Methods

Forty one young healthy female Chinese subjects who were taking part in a larger study of determinants of bone mass were asked to collect 24-h urine samples while they were on their usual intake of nutrients. Urine samples were collected into bottles containing hydrochloric acid and the volume of the urine samples was measured and aliquots were stored at −20°C for assay of dopamine, digoxin-like immunoreactive substance, sodium, potassium and calcium. Sodium, potassium and calcium concentrations in urine were measured by standard methods in an automated analyser. Urinary free dopamine was extracted onto alumina using the method of Davidson et al. [19] and quantitated by high performance liquid chromatography using electrochemical detection as described previously [20]. The intra-assay and inter-assay coefficient of variation were 1.3% and 8.7%, respectively. The mean recovery of urinary free dopamine was 91.1% and the detection limit was 3 nmol/l. Urinary digoxin-like immunoreactive substance was measured by immunoassay using a commercial kit (CEDIA Digoxin, Microgenic, Concord, CA, USA) adapted to a centrifugal analyser (Cobas Fara, Hoffmann La Roche, Basle, Switzerland). The limit of detection was 0.08 nmol/l and the inter-assay coefficient of variation was better than 7.5%. Stepwise multiple regression analysis was used to examine the relationship between these variables and others. This method searches through all the variables and adds in, at each step, the variable that increases the coefficient of multiple correlation to the greatest extent. The process continues until either the increase in the coefficient is non-significant or all the variables are included.

Results

Table 1 gives the mean, S.D. and range for the variables measured in the study. The age range of the group was 18–23 years. The mean dopamine excretion was 1463 μmol/day and the excretion of digoxin-like immunoreactive substance varied from 0.42–1.98 nmol/day. Table 2 shows the correlation between the variables. Sodium excretion was significantly related to the excretion of potassium, calcium, free dopamine and digoxin-like immunoreactive substance. Potassium excretion was not related to calcium, dopamine or digoxin-like immunoreactive substance. Digoxin-like immunoreactive substance was also significantly correlated with calcium excretion. Stepwise multiple regression analysis with dopamine as the dependent variable and sodium, potassium, calcium and digoxin-like immunoreactive substance as independent variables showed that sodium contributed significantly to dopamine excretion. Of the variation in dopamine excretion 29.7% was accounted for by variation in sodium intake. When a similar analysis was done with digoxin-like immunoreactive substance as the dependent variable, only calcium excretion was significantly related ($r = 0.338$, $P < 0.05$) and 11.4% of the variation in digoxin-like immunoreactive substance was accounted for by calcium excretion.

TABLE 1

Age and urinary excretion of sodium, potassium, calcium, dopamine and DLS in 41 healthy young females.

	Mean	S.D.	Range
Age (years)	19.1	1.1	18–23
Sodium excretion (mmol/day)	132	52.8	54–302
Potassium excretion (mmol/day)	39	12.2	18.4–75.2
Calcium excretion (mmol/day)	2.83	1.21	0.8–6.2
Dopamine excretion (μmol/day)	1463	304	920–2134
DLS (nmol/day)	1.14	0.408	0.42–1.98

TABLE 2

Correlation between variables.

	Sodium	Potassium	Calcium	Dopamine
Potassium	0.514 (<0.001)		—	
Calcium	0.348 (<0.03)	N.S.	—	
Dopamine	0.545 (<0.0005)	N.S.	N.S.	—
DLS	0.359 (<0.02)	N.S.	0.345 (<0.03)	N.S.

Figures in parentheses give the *P*-value.

Discussion

The 24-h urinary excretion of potassium in this group of Hong Kong Chinese is lower than that in many parts of the world, but is similar to that reported from China [21] and Hong Kong [22]. The mean excretion of calcium in this group was 2.83 mmol/day and it is also lower than that reported from Western Countries [21]. The 24-h excretion of calcium in the steady state reflects intestinal absorption of calcium [23] and the amount of calcium absorbed is directly related to the intake of calcium [24]. The low calcium excretion among the Chinese reflects the low dietary intake of calcium [25,26].

Dopamine has been shown to be a natriuretic hormone [16] and the excretion of dopamine and sodium are correlated in normotensive Caucasians [18] and Japanese [15]. We have previously found a significant relationship between sodium excretion and dopamine excretion in healthy Chinese men and women (Chan, Ho, Critchley and Swaminathan, unpublished observations). Our findings confirm this and furthermore we show that the major determinant of dopamine among the electrolytes is sodium (Table 2). Further studies are necessary to elucidate the relationship between dopamine and sodium excretion.

Increased dietary salt stimulates the release of a Na^+, K^+-ATPase inhibitor [13,14]. This inhibitor can be detected by inhibition of sodium pump activity of intact cells, inhibition of enzyme activity or by immunoassay using antibodies raised against digoxin [14,27].

Using an immunoassay we detected digoxin-like immunoreactive substance in all urine samples and the excretion of digoxin-like immunoreactive substance was directly related to the excretion of sodium ($r = 0.359$, $P < 0.02$) and to calcium ($r = 0.345$, $P < 0.03$). However, stepwise multiple regression analysis showed that only calcium excretion was positively correlated with excretion of digoxin-like immunoreactive substance. This lack of significant association between sodium excretion and digoxin-like immunoreactive substance agrees with the hypothesis of De Wardener and MacGregor [11]. If in normal subjects there is no abnormality in mobilization of dopamine, sodium intake is unlikely to stimulate the production of Na^+, K^+-ATPase inhibitor. On the other hand, calcium intake in some manner may stimulate the release of this inhibitor. In the rat, increased calcium intake reduced the plasma concentration of digoxin-like immumoreactive substance [14]. However, the relationship between digoxin-like immunoreactive substance and calcium excretion in this study was a positive one. If low calcium intake is a factor in the pathogenesis of hypertension [1] then one would expect a negative relationship. Further detailed studies are required to understand this complex interrelationship.

We conclude that in young healthy subjects dopamine excretion is determined at least partly by sodium intake and the excretion of digoxin-like substance is independent of sodium intake.

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THE EFFECT OF SALT LOADING ON THE URINARY EXCRETION OF DOPAMINE AND SODIUM TRANSPORT INHIBITOR IN THE RAT

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ABSTRACT

1. The temporal relationship between the excretion of dopamine and sodium transport inhibitor (STI) during salt loading was examined in the rat.

2. Urine samples were collected before and during salt loading (given as 18 g/l NaCl solution to replace drinking water) for the measurement of sodium, creatinine, dopamine and STI in 6 female rats. Dopamine was measured by HPLC and STI was extracted and measured by its ability to inhibit purified Na^+, K^+-ATPase enzyme.

3. Urinary sodium and STI (expressed in relation to creatinine) on day 1 of salt loading were 4.6 and 4.2 times respectively of the control values.

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Urinary excretion of dopamine did not increase significantly until day 2 when it was 21% higher.

4. The excretion of STI paralleled that of sodium excretion whereas the excretion of dopamine lagged behind.

5. We conclude that salt loading increases STI and dopamine and that the increase in STI precedes that of dopamine.

INTRODUCTION

The mechanism by which high salt intake or expansion of ECF by saline infusion causes natriuresis has been extensively studied and several natriuretic factors have been identified. These include atrial natriuretic peptide, renal prostaglandins, kallikreins, renal dopamine and sodium transport inhibitor (STI, Ouabain-like factor).

As it has been shown that urinary excretion of dopamine increases during salt loading [1-3] and dopamine infusion causes natriuresis [4], it has been suggested that renal dopamine participates in the regulation of sodium balance [5]. It has also been demonstrated that the natriuresis induced by volume expansion is mediated at least in part by a circulating sodium transport inhibitor (STI) [6-12]. Furthermore dopamine and STI are implicated in the

pathogenesis of salt-sensitive hypertensive. It has been suggested that in hypertensives renal dopamine production is impaired [13] and STI increases to maintain salt balance. Increased STI levels may lead to contraction of the vascular smooth muscle [14]. Although dopamine [2] and STI [11,15] have been shown to increase during salt-loading, the role of these substances in the natriuresis of salt loading is not clear. The purpose of this study was to examine the temporal relationships between the changes in the excretion of sodium, dopamine and STI during salt loading in the rat.

MATERIALS AND METHODS

Six female Sprague-Dawley rats weighing 250 g were studied. They were fed ad libitum throughout the study with normal rat chow containing 0.2% sodium chloride. The animals were placed in individual metabolic cages and given tap water to drink for 4 days. 24 hr urine sample were collected for two days (days -2 and -1). On day 1 the tap water was changed to 18 g/l NaCl solution and urine collection was continued for another 7 days. Urine samples were collected in 6.0M HCl and after recording the volume, they were stored at -70°C.

Urinary sodium and creatinine concentrations were measured by indirect ion-selective electrode and Jaffe reaction, respectively on a Beckman Astra-8 Clinical chemistry analyzer (Beckman Instrument, CA, USA). The interassay coefficients of variation for both assays were less than 3%.

Urinary free dopamine was determined as described previously [16]. Briefly, dopamine was extracted onto alumina, eluted with 0.1 M HCl, washed with ethylacetate as described by Davidson and Fitzpatrick [17] and quantitated by high performance liquid chromatography with electrochemical detector based on a method described by Weicker et al [18]. Intra-assay and inter-assay coefficient of variation were 1.3% and 8.7% respectively. The mean recovery was 91% and the detection limit was 3 nM.

Urinary STI was extracted using Waters Sep-Pak C18 cartridges (Millipore, Milford, MA, USA) and quantitated by its ability to inhibit purified dog kidney Na^+, K^+ ATPase activity (Sigma, St Louis, MO, USA). Briefly, Sep-Pak cartridges were first activated by washing with methanol and water. 300 ul of sample was applied and was allowed to equilibrate

with the C18 resin in the cartridge for 2 min before washing twice with 10 ml of water to remove salts and other interfering substances. The equilibration step was found necessary to improve the precision of the extraction step. STI was eluted twice with 1 ml of methanol. The methanol eluate was evaporated to dryness and the residue was reconstituted in 100 ul Tris buffer, pH 7.4. These reconstituted eluates contained no measurable dopamine. The reconstituted extract was then incubated with a 250 U/l of purified dog kidney Na^+, K^+-ATPase solution in the presence of 100 nM of sodium and 5 nM of ATP at 37°C for 2 hours. The reaction was terminated by immersing the reaction mixture in ice. The residual ATPase activity was determined by an enzyme coupled reaction as described previously [19]. Ouabain in Tris buffer, ranging from 5 nM to 50 nM, were used as standards and the results are expressed as nM ouabain equivalent. Interassay coefficient of variation ranged from 18% to 3% over the standard concentrations.

Excretion of sodium, dopamine and STI were related to creatinine in order to compensate for any loss of urine during collection and for any differences in lean body mass during the study.

Table 1 Effect of salt loading on the excretion of sodium, dopamine and sodium transport inhibitor (STI) in rats.

Day	Urine volume (mmol/d)	Sodium/Cr (mmol/mmol)	Dopamine/Cr (nmol/mmol)	STI/Cr (nmol ouabain/mmol)
-2	10.0 ± 1.9	37 ± 3.3	240 ± 22.0	0.46 ± 0.15
-1	9.8 ± 3.0	39 ± 5.5	211 ± 21.3	0.40 ± 0.15
1	27.5 ± 8.3*	174 ± 48.0**	246 ± 34.6	1.80 ± 0.30**
2	29.2 ± 11.2*	210 ± 46.4**	273 ± 31.9*	2.67 ± 0.452**
3	24.7 ± 10.6*	203 ± 33.5**	329 ± 45.7**	2.61 ± 0.436**
4	23.3 ± 7.0*	192 ± 29.0**	336 ± 56.1**	2.83 ± 1.10**
5	17.1 ± 5.9*	190 ± 13.8**	308 ± 53.2**	3.74 ± 1.07**
6	27.6 ± 8.3*	186 ± 17.6**	305 ± 39.0**	2.92 ± 0.957**
7	19.2 ± 5.8*	206 ± 18.2**	371 ± 46.7**	2.76 ± 0.642**

Results are mean ± SD, n=6

*, ** Significantly different from days -1 and -2

For statistical analysis the mean values of day -1 and -2 were used to represent the excretion before salt-loading. The data were subjected to analysis of variance and where the null hypothesis was rejected. Scheffe's comparisons were performed on the means; $P<0.05$ was considered significant.

RESULTS

Table 1 summaries the results. Urine sodium excretion on day 1 was significantly higher than pre-salt loading and the sodium excretion reached a peak

on day 2 and thereafter remained relatively stable. Urine dopamine excretion on day 1 was 9% higher than basal value but the difference was not significant. On day 2 the urinary excretion of dopamine was 21% higher than the mean before the salt loading ($p<0.01$). Urine dopamine excretion reached a peak on day 4 and remained high there after. On the other hand, urine excretion of STI on day 1 was 4.2 fold higher than basal values.

As can be seen from Table 1 the excretion of STI paralleled the rise in sodium whereas dopamine excretion lagged behind.

DISCUSSION

High salt intake has been shown to cause an increase in excretion of dopamine [2,3] and STI [15]. The evidence for the release of STI during salt loading comes from direct measurement of the inhibitory activity [15,20] or from indirect measurement of Na^+, K^+-ATPase activity of circulating cells [21]. We have attempted to investigate the relative importance of dopamine and STI to the natriuresis of salt-loading by examining the temporal relationships. Our results confirm previous observations that high salt intake causes an increase

in dopamine excretion [2]. The magnitude of increase (approximately 50% on day 4) is similar to that reported by others [1-3].

Our results also confirm that high salt diet causes an increase in STI [7,11,15,20]. However as far as we are aware there has been no reports on the temporal relationship between dopamine and STI during salt loading. The results presented here show that STI closely followed the excretion of sodium (Table 1) where as the excretion of dopamine lagged behind by a day. Furthermore unless the natriuretic effect of dopamine is several fold higher than other natriuretic factors, the observed increase in dopamine excretion may not be quantitatively adequate to explain the natriuresis. However, it is possible that local concentration of dopamine at the tubules may be high enough to cause this degree of natriuresis.

These observations on dopamine could be interpreted in two ways. (a) Dopamine is not important in the natriuresis of salt loading and that the increased excretion of dopamine is secondary to the excretion of sodium. Observations such as lack of increase in dopamine excretion during head out water

immersion (Ho, O'Hare, Bison & Swaminathan - unpublished results) and the lack of effect of carbidopa, a DOPA decarboxylase inhibitor on the natriuresis induced by saline infusion [23] support this theory. However, there are other observations which support a natriuretic role for dopamine. For instance, administration of Carbidopa to healthy subjects reduced sodium excretion suggesting that dopamine has a tonic role in the excretion of sodium [13,24]. Dopaminergic blockade by the use of dopamine receptor antagonist metoclopramide abolished the natriuretic response to saline infusion [25]. (b) Dopamine may be important in maintaining the natriuresis initiated by other factors such as STI or ANP.

The results reported here also strongly suggest a role for STI in the natriuresis and further work is needed to define the relative roles of STI, dopamine, ANP and other natriuretic factors.

We conclude that salt loading in the rat causes an increase in STI and dopamine and that the increase in STI precedes that of dopamine.

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